EXHIBIT 34

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

BIOHAVEN THERAPEUTICS LTD. and YALE UNIVERSITY, Plaintiffs, v. AVILAR THERAPEUTICS, INC., a Delaware Corporation, RA CAPITAL MANAGEMENT GP, LLC, a Delaware Corporation Defendants.	C.A. No. 23-cv-328-CFC-SRF **HIGHLY CONFIDENTIAL PURSUANT TO THE PROTECTIVE ORDER (D.I. 65)**

PLAINTIFF YALE UNIVERSITY'S AMENDED AND SUPPLEMENTAL RESPONSES AND OBJECTIONS TO DEFENDANTS' FIRST SET OF INTERROGATORIES (1-12)

Pursuant to Rule 26 and 33 of the Federal Rules of Civil Procedure and the applicable Local Rules of Civil Practice and Procedure of the United States District Court for the District of Delaware, Plaintiff Yale University ("Yale"), by and through its counsel of record, hereby serves the following amended and supplemental responses and objections to Defendants' First Set of Interrogatories (1-12), served on July 19, 2023.

PRELIMINARY STATEMENT

Yale provides these responses and objections to Defendants' First Set of Interrogatories based on the information presently available to Yale. Discovery is ongoing, and future discovery and investigation may reveal additional facts or information and/or may establish entirely new factual conclusions, which may lead Yale to change its responses to these Interrogatories. Yale reserves the right to amend and/or supplement its responses should it discover other responsive information. Yale also reserves the right to use subsequently discovered information in depositions, at trial, and in

support of or opposition to any motion, petition, or other proceeding in this matter. Yale makes each of these responses solely for the purpose of, and in relation to, this case.

Yale does not concede that any portion of its responses to these Interrogatories is admissible at trial or in connection with any non-discovery motion, petition or proceeding. Yale reserves all rights to object to the introduction of any portion of these responses—or any information or document produced pursuant to them—at trial or any non-discovery motion, petition or proceeding.

Finally, Yale's objections and responses to Defendants' Interrogatories are made in good faith. Where appropriate Yale has asserted specific objections in response to specific Interrogatories. Nevertheless, Yale with its responses to these Interrogatories does not waive but expressly preserves all objections on grounds of privilege, work product protection, right of privacy or any other such privilege or immunity. Yale further does not waive but expressly preserves its right to protect against the inappropriate and inadvertent disclosure of confidential and proprietary information belonging to Yale, Plaintiff Biohaven Therapeutics Ltd. ("Biohaven"), or third parties. Yale's good faith responses to these Interrogatories should not be read to imply that Yale has in any way waived such objections or protections. To the extent Defendants attempt to interpret these Interrogatories in a way that would require Yale to specifically invoke any of these objections or protections, Yale reserves the right to do so.

GENERAL OBJECTIONS AND RESPONSES

1. The following general objections and responses are incorporated into Yale's responses to each of the Interrogatories and shall have the same force and effect as if set forth in each of Yale's specific reasons below.

2. Yale objects to the Interrogatories to the extent they seek to impose obligations inconsistent with, beyond, or in addition to those imposed by the Federal Rules of Civil Procedure the Local Rules of Civil Practice and Procedure of the United States District Court for the District of

Delaware, Chief Judge Connolly's individual practices, and any other court order or stipulation governing discovery in this case. Yale will respond in conformity with the Electronically Stored Information ("ESI") Order entered into by the parties (D.I. 68).

3. By responding to the Interrogatories, Yale does not concede that any information requested is relevant to this action or admissible at trial and does not waive any objection concerning competency, relevancy, materiality, or admissibility. Yale reserves the right to object to further discovery on the subject matter of any of the Interrogatories.

4. Yale objects to the Interrogatories to the extent they seek information protected by the attorney-client privilege, the attorney work product doctrine, common interest privilege, and/or any other applicable privileges. Yale will not provide any privileged and/or protected information. Any disclosure of privileged information would be inadvertent and should not be deemed a waiver of privilege. Yale does not intend to log information generated after the filing of this lawsuit.

5. Yale objects to these Interrogatories to the extent they seek expert work, testimony, or opinions. Yale will produce expert materials consistent with the applicable Federal and Local Rules and the Court's scheduling order.

6. Yale objects to these Interrogatories to the extent they mischaracterize the facts or law. Yale's response to any interrogatory does not waive any right by Yale to take a factual or legal position or to contest any of Defendants' factual or legal positions.

7. Yale objects to the Interrogatories to the extent that they call for disclosure of personal, commercially sensitive, or third-party confidential information. Yale will only disclose this information pursuant to the Protective Order (D.I. 65) and consistently with any confidentiality agreement with any third-party.

OBJECTIONS TO DEFINITIONS AND INSTRUCTIONS

1. Yale objects to the definition of "Yale" as overly broad, unduly burdensome, and encompassing irrelevant information to the extent it includes individuals, categories of individuals, or entities who have no involvement with the Asserted Trade Secrets or the allegations in this lawsuit, including "professors," "Dr. Crews," and the "Crews Lab." Yale will not independently provide information related to these individuals or entities, including "professors," "Dr. Crews," or the "Crews Lab." Yale further objects to this definition to the extent it encompasses Yale students (undergraduate or graduate) who are not also employed by Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit. Yale will not independently search for or provide information about students who are not also employed by Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit.

2. Yale objects to the definitions of "Plaintiffs," "You," and "Your" for the same reasons it objects to the definition of "Yale."

3. Yale objects to the definition of "Spiegel Lab" to the extent it encompasses Yale students (undergraduate or graduate) who are not also employees of Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit. Yale will not independently search for or provide information about students who are not also employed by a Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit.

4. Yale objects to the definition of "Dr. Crews" because Dr. Crews has no relevance to this litigation. Yale will not independently provide information related to "Dr. Crews."

5. Yale objects to the definition of "Crews Lab" because Dr. Crews and the Crews Lab have no relevance to this litigation. Yale will not independently provide information related to "Dr. Crews" or the "Crews Lab." Yale further objects to this definition to the extent it encompasses Yale students (undergraduate or graduate) who are not also employees of Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit. Yale will not independently search for or provide information about students who are not also employed by a Yale in a position related to the Asserted Trade Secrets or the allegations in this lawsuit.

6. Yale objects to the definition of "Trade Secret" and "Trade Secrets" to the extent it is inconsistent with the Defend Trade Secrets Act or the Delaware Uniform Trade Secrets Act.

7. Yale objects to the term "identify with precision and specificity each and every alleged Trade Secret." Yale responds to Defendants' interrogatories in compliance with the applicable rules for identifying trade secrets.

8. Yale objects to Instruction No. 9 to the extent it seeks to impose obligations inconsistent with, beyond, or in addition to those imposed by the applicable federal, local, and any other rules.

9. Yale's understands and uses "Asserted Trade Secrets" in these responses and objections to refer to Plaintiffs' HIGHLY CONFIDENTIAL Trade Secret Identification, dated October 27, 2023 (also referred to as "TSID").

RESPONSES AND OBJECTIONS TO INTERROGATORIES

INTERROGATORY NO. 1:

Separately for each Defendant, identify with precision and specificity each and every alleged Trade Secret that Plaintiffs contend that Defendant unlawfully acquired, used, or disclosed.[1]

[1] The phrase "identify with precision and specificity each and every alleged Trade Secret" as used in Interrogatory No. 1 means to provide a numbered list of each individual such alleged Trade Secret (with each number constituting a separate Trade Secret) with a specific description of each such

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the footnote included with the Interrogatory (footnote 1), which requests a numbered list of "individuals" even though the interrogatory relates to "Trade Secrets."

[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

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alleged Trade Secret in such a manner that the exact identity, scope, boundaries, constitutive elements, and content of each such alleged Trade Secret are fully disclosed in writing, in contrast to an agglomerated set of conclusory phrases that does not separately list and describe each such alleged Trade Secret, in contrast to a mere list of documents or file names, and with precision far above that required by any applicable pre-discovery Trade Secret claim identification requirement. If Plaintiffs contend that a particular combination of information constitutes an alleged Trade Secret, "identify with precision and specificity each and every alleged Trade Secret" shall also mean to identify the unique combination of constitutive elements that Plaintiffs contend together constitutes an alleged Trade Secret.

INTERROGATORY NO. 2:

Separately for each Asserted Trade Secret, identify with precision and specificity every person and entity who participated in any way in the development or creation of the Asserted Trade Secret, the date(s) of such development or creation, the time and financial investment spent on such development or creation, and any documents memorializing or regarding such development or creation.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of information that is shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrase "every person," "participated in any way," "development or creation," "time and financial investment," and "memorializing or regarding" as vague and ambiguous, overly broad, unduly burdensome, and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory to the extent it requires Yale to provide its damages case ahead of what is required by Federal Rules of Civil Procedure and the applicable Local Rules of Civil Practice and Procedure.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

Yale's discovery efforts and investigation are ongoing. Yale reserves the right to supplement these responses.

INTERROGATORY NO. 3:

Separately for each Asserted Trade Secret, identify with precision and specificity each and every measure taken by each Plaintiff to safeguard and keep secret the Asserted Trade Secrets from their creation until the present time, including the date(s) such measures were employed, all witnesses to such measures and any documents constituting, evidencing or memorializing such measures.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrases "each and every measure," "safeguard and keep secret," "all witnesses," and "evidencing or memorializing" as vague and ambiguous, overly broad, unduly burdensome, and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

Yale's discovery efforts and investigation are ongoing. Yale reserves the right to supplement these responses.

INTERROGATORY NO. 4:

Separately for each Asserted Trade Secret, identify with precision and specificity the independent economic value, if any, to each Plaintiff from each such Asserted Trade Secret not being generally known to, and not being readily ascertainable through proper means by, another person who can obtain economic value from the disclosure or use of the information.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale further objects to the phrases "independent economic value" and "not being readily ascertainable through proper means" as vague and ambiguous. Yale further objects to this request to the extent it prematurely seeks expert testimony.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory because the relevant information regarding the economic value of the Asserted Trade Secrets is within the

possession of Defendants. Moreover, Defendants have yet to produce internal correspondence at RA Capital and Avilar during the period of time when negotiations ended and Avilar filed its patent applications incorporating Plaintiffs' Asserted Trade Secrets. Yale will supplement its response to this interrogatory upon Defendants' production of documents in response to Plaintiffs' requests.

Subject to and without waiving the general and specific objections, Yale responds as follows: [redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 5:

Separately for each Asserted Trade Secret, identify with precision and specificity every person or entity to whom Plaintiffs have ever disclosed such Asserted Trade Secret at any time (i.e.,

before or after the initiation of this lawsuit), including the date(s) of all such disclosures, all witnesses to each such disclosure and any documents constituting, evidencing or memorializing each such disclosure.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrases "every person or entity to whom plaintiffs have ever disclosed," "all witnesses," and "evidencing or memorializing" as vague and ambiguous, overly broad and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound. Yale further objects to this Interrogatory to the extent it seeks information covered by confidentiality obligations to a third party.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

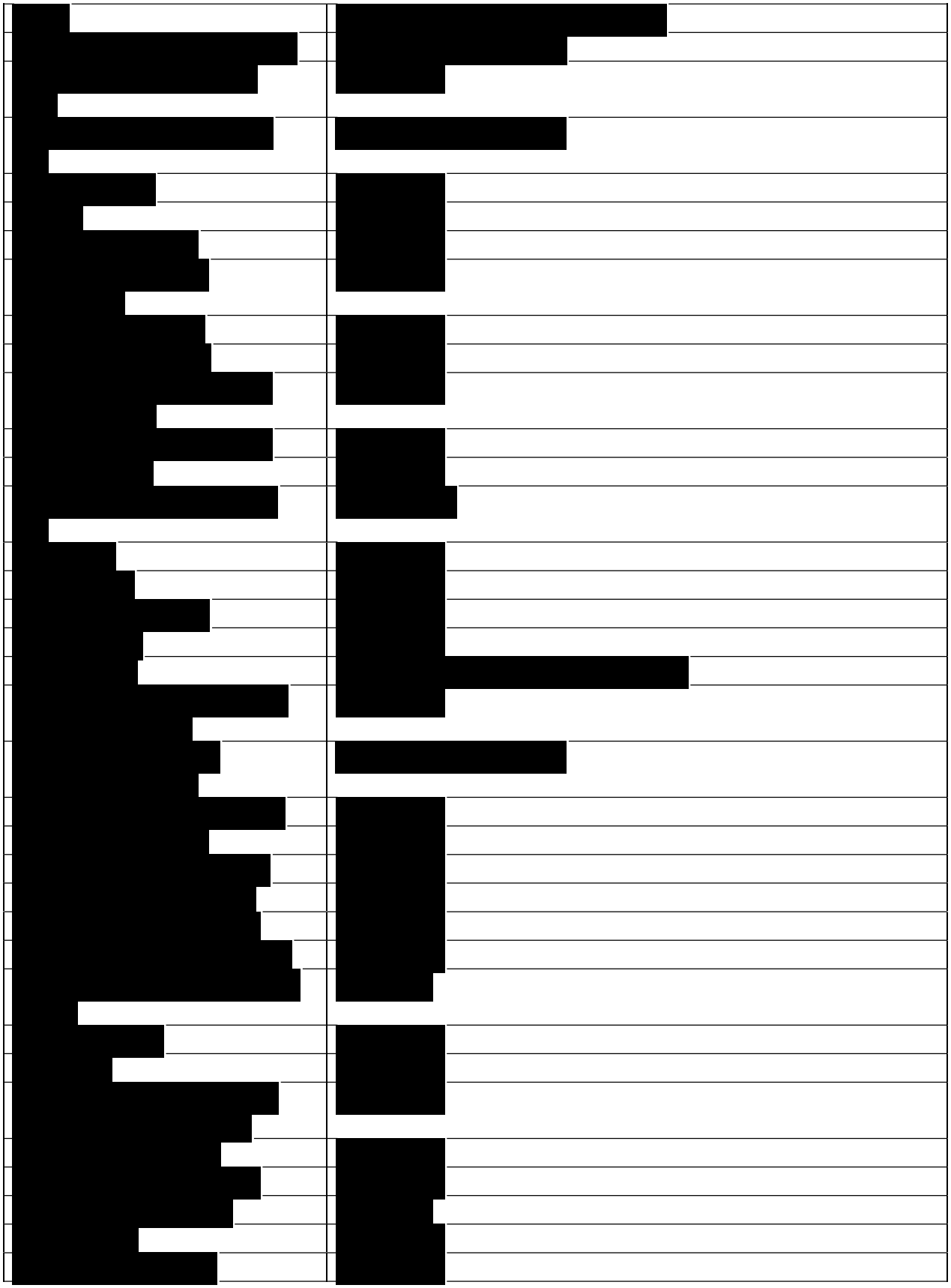
SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome.

Subject to and without waiving the general and specific objections, Yale answers as follows:

[redacted]

[redacted]



[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 6:

Separately for each Asserted Trade Secret, identify with precision and specificity how Plaintiffs contend each Defendant received or otherwise obtained each Alleged Trade Secret, including the date(s) when Plaintiffs contend each Defendant received or obtained each Alleged Trade Secret, the exact means by which each Defendant received or obtained each Alleged Trade Secret, and any document constituting, evidencing or memorializing such receipt.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome,

and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrases "exact means" and "evidencing or memorializing" as vague and ambiguous, overly broad, and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory because the relevant information regarding Defendants' improper use and disclosure of the Asserted Trade Secrets is within the possession of Defendants, which is the subject of Plaintiffs' discovery requests and which Defendants have not yet produced.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 7:

Separately for each Asserted Trade Secret that Plaintiffs contend Defendants used without Plaintiffs' authorization or consent, identify with precision and specificity each such alleged use and which Defendant(s) made the alleged use, including the exact information allegedly used, all date(s) of each such alleged unauthorized use, all place(s) of such unauthorized use, the identity of all person(s) who made each such unauthorized use, all witnesses to each such unauthorized use, the exact manner in which each such unauthorized use was effectuated and any documents constituting, evidencing or memorializing each such unauthorized use.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrases "exact information," "exact manner," "all person(s)," "all witnesses," "all date(s)," "all place(s),' and "evidencing or memorializing" as vague and ambiguous, overly broad and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in the case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory because

the relevant information regarding Defendants' improper use and disclosure of the Asserted Trade Secrets is within the possession of Defendants, which is the subject of Plaintiffs' discovery requests and which Defendants have not yet produced.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

Defendants' unlawful uses and disclosures of the Asserted Trade Secrets constituted breaches of the confidential disclosure agreement it entered with Yale. Yale disclosed the Asserted Trade Secrets for the sole purpose of enabling the parties to evaluate a possible contractual arrangement between themselves. Complaint Ex. 1 ¶ 2. Defendants were bound by the Confidential Disclosure Agreement to not make use of the Asserted Trade Secrets for any other purpose without Yale's prior written consent, which Defendants never sought or received. Complaint Ex. 1 ¶ 8.

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 8:

Separately for each Asserted Trade Secret that Plaintiffs contend Defendants disclosed without Plaintiffs' authorization or consent, identify with precision and specificity each such alleged disclosure and which Defendant(s) made the alleged disclosure, including the exact information allegedly disclosed, all date(s) of each such alleged unauthorized disclosure, all place(s) of such unauthorized disclosure, the identity of all person(s) who made each such unauthorized disclosure,

all witnesses to each such unauthorized disclosure, the exact manner in which each such unauthorized disclosure was effectuated and any documents constituting, evidencing or memorializing each such unauthorized disclosure.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrases "exact information," "exact manner," "all person(s)," "all witnesses," "all date(s)," "all place(s)," and "evidencing or memorializing" as vague and ambiguous, overly broad and disproportionate to the needs of this case. Yale further objects to this Interrogatory as compound.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory because the relevant information regarding Defendants' improper use and disclosure of the Asserted Trade Secrets is within the possession of Defendants, which is the subject of Plaintiffs' discovery requests and which Defendants have not yet produced.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

Defendants' unlawful uses and disclosures of the Asserted Trade Secrets constituted breaches of the confidential disclosure agreement it entered with Yale. Yale disclosed the Asserted Trade Secrets for the sole purpose of enabling the parties to evaluate a possible contractual arrangement between themselves. Complaint Ex. 1 ¶ 2. Defendants were bound by the Confidential Disclosure Agreement to not make use of the Asserted Trade Secrets for any other purpose without Yale's prior written consent, which Defendants never sought or received. Complaint Ex. 1 ¶ 8.

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 9:

Separately for each Asserted Trade Secret, identify with precision and specificity all facts in support of Your contention that such Asserted Trade Secret was neither generally known nor readily ascertainable by proper means.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to the phrase "readily

ascertainable by proper means" as vague and ambiguous.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome. Yale further objects to this Interrogatory because relevant information regarding Defendants' improper use and disclosure of the Asserted Trade Secrets is within the possession of Defendants, which is the subject of Plaintiffs' discovery requests and which Defendants have not yet produced.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 10:

Separately for each Asserted Trade Secret, identify with precision and specificity whether such Asserted Trade Secret was ever included, disclosed or described in any Spiegel Patent, Yale Patent or Biohaven Patent, and identify with precision and specificity any Spiegel Patent, Yale Patent or Biohaven Patent which includes, discloses or describes such Asserted Trade Secret and where (by page number and, if applicable, line number) the Asserted Trade Secret is included, described or disclosed.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale will respond to this Interrogatory in compliance with the applicable rules for identifying trade secrets. Yale further objects to this Interrogatory to the extent the information is equally obtainable by Defendants.

Subject to and without waiving the general and specific objections, Yale will identify the Trade Secrets according to the scheduling order in this case.

SUPPLEMENTAL RESPONSE:

Yale further objects to the phrase "Separately for each Asserted Trade Secret" as vague and ambiguous, overly broad, and unduly burdensome.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 11:

With respect to Plaintiffs' cause of action for breach of contract, identify with precision and specificity all facts that Plaintiffs contend constitute a breach of the Confidential Disclosure Agreement, including the date(s) of each such breach, the specific provision(s) of the CDA breached, the conduct that constituted each such breach, the person(s) who participated in each such breach, the witness(es) to each such breach, and any documents constituting, evidencing or memorializing each such breach.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by

the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects to the phrase "identify with precision and specificity" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale further objects because Defendants have refused to provide any substantive response to any of Plaintiffs' Interrogatories.

Subject to and without waiving the general and specific objections, Yale is willing to meet and confer about this Interrogatory.

SUPPLEMENTAL RESPONSE:

Yale further objects to this Interrogatory because the relevant information regarding Defendants' improper disclosure of the Asserted Trade Secrets is within the possession of Defendants. Moreover, Defendants have yet to produce internal correspondence at RA Capital and Avilar during the period of time when negotiations ended and Avilar filed its patent applications incorporating Plaintiffs' Asserted Trade Secrets. Yale will supplement its response to this interrogatory upon Defendants' production of documents in response to Plaintiffs' requests.

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

[redacted]

Defendants' unlawful uses and disclosures of the Asserted Trade Secrets constituted breaches of the confidential disclosure agreement it entered with Yale, including sections 2, 4, 6, 8, and 9. Yale disclosed the Asserted Trade Secrets for the sole purpose of enabling the parties to evaluate a possible contractual arrangement between themselves. Complaint Ex. 1 ¶ 2. Defendants were bound by the Confidential Disclosure Agreement to not make use of the Asserted Trade Secrets for any other purpose without Yale's prior written consent, which Defendants never sought or received. Complaint Ex. 1 ¶ 8.

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

INTERROGATORY NO. 12:

State with specificity and precision the legal and factual bases for Your contention that the information shared by Dr. Spiegel or Yale with RA Capital pursuant to the Confidential Disclosure Agreement was not known to RA Capital prior to the disclosure by Dr. Spiegel or Yale, did not become publicly known through no fault or omission attributable to RA Capital, was not given to RA Capital by a third party under no obligation of confidentiality to Yale, or was not independently developed by RA Capital without the aid, application or use of the confidential information shared by Dr. Spiegel or Yale.

RESPONSE:

Yale incorporates by reference the General Objections. Yale further objects to this Interrogatory to the extent that it seeks discovery of documents that are shielded from disclosure by the attorney-client privilege, the work product doctrine, or any other applicable privilege or protection, including information covered by any common interest privilege. Yale further objects

to the phrase "[s]tate with specificity and precision" as vague and ambiguous, unduly burdensome, and disproportionate to the needs of the case. Yale further objects to this Interrogatory to the extent the information is equally obtainable by Defendants. Yale further objects to this Interrogatory because it is compound. Yale further objects to this Interrogatory because Defendants have refused to provide any substantive response to any of Plaintiffs' Interrogatories.

Subject to and without waiving the general and specific objections, Yale is willing to meet and confer about this request.

SUPPLEMENTAL RESPONSE:

Subject to and without waiving the general and specific objections, Yale responds as follows:

[redacted]

[redacted]

[redacted]

Yale's discovery efforts and investigation are ongoing. Accordingly, Yale reserves the right to supplement these responses.

Dated: December 20, 2023

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CERTIFICATE OF SERVICE

I, Brian E. Farnan, hereby certify that on December 20, 2023, a copy of Plaintiff Yale University's Amended Responses and Objections to Defendants' First Set of Interrogatories (1-12) was served on the following as indicated:

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EXHIBIT 35

Biohaven Advances Development of the MoDE Platform Technology Licensed From Yale University

biohaven

NEWS PROVIDED BY
Biohaven Ltd. →
Oct 25, 2022, 16:30 ET

- *Biohaven advances development of its extracellular target degrader platform technology (MoDEs™) for therapies across a variety of diseases including neuroscience, immunology and oncology*
- *Through its continued collaboration with Yale University in the laboratory of Professor David Spiegel and Biohaven Labs, Biohaven has made further innovations in this ground-breaking technology with new patent applications covering additional targets and functionality*
- *In-licensed from Yale University in 2021, the MoDE platform is based on patent applications filed by Yale in 2018 and 2019*

NEW HAVEN, Conn., Oct. 25, 2022 /PRNewswire/ -- Biohaven Ltd. (NYSE: BHVN; "Biohaven") today announced advancements in the development of its MoDE extracellular target degrader platform technology licensed from Yale University for various disease indications, including, but not limited to, neurological disorders, cancer, infectious and autoimmune diseases.

In 2021, Biohaven entered into a worldwide, exclusive license agreement to develop and commercialize the MoDE platform based on ground-breaking research conducted in the laboratory of Professor David Spiegel at Yale University. Under the license agreement, Biohaven

acquired exclusive, worldwid[redacted]irected to its MoDE technology, which pertains to the clearance of disease-causing proteins and other biomolecules by targeting them for lysosomal degradation using multi-functional molecules.

Professor Spiegel commented: "Targeted protein degradation (TPD) has emerged as a promising and exciting therapeutic strategy, however, the majority of existing TPD technologies to date rely on the ubiquitin-proteasome system that targets intracellular proteins. To address this limitation, our lab developed MoDEs, a class of modularly designed, bifunctional synthetic molecules, which are capable of mediating the degradation of extracellular proteins through the asialoglycoprotein receptor (ASGPR). MoDE molecules mediate the formation of a ternary complex between a target protein and the ASGPR, which is expressed primarily on hepatocytes, and the target protein is then endocytosed and degraded by lysosomal proteases. The MoDE platform is differentiated from existing approaches, such as Proteolysis Targeting Chimeras (PROTAC), in that it does not rely on ubiquitin ligases, and allows for a broad range of targets to be degraded."

Through the novel approach, Professor Spiegel demonstrated the modularity of the MoDE technology by synthesizing bifunctional molecules that induce the degradation of both antibody and pro-inflammatory cytokine proteins. When initially published, the data obtained by the Spiegel Lab represented the first experimental evidence that non-proteinogenic, synthetic molecules could be employed for the TPD of extracellular proteins both *in vitro* and *in vivo*.

The MoDE platform discovered at Yale University is described in a peer-reviewed publication entitled "Bifunctional Small Molecules that Mediate the Degradation of Extracellular Proteins" (*Nature Chemical Biology* 2021, 17, 947–953) and in various Yale University patent applications, including applications published as WO 2019/199621 and WO 2019/199634, both of whom are based on provisional applications filed in 2018. Subsequent to the Yale patent filings, others have referred to such multifunctional molecules for extracellular protein degradation as "ASGPR Targeting Chimeras" (ATACs) (see, for example, https://endpts.com/an-ra-backed-startup-atacs-a-novel-challenge-looking-to-spur-protein-degradation-outside-the-cell) or "Lysosome-Targeting Chimeras" (LyTACs) (see, for example, https://endpts.com/going-where-protacs-cant-versant-unveils-50m-bet-on-carolyn-bertozzis-lytac-tech-with-a-seasoned-biotech-exec-at-the-helm). Both Yale and Biohaven continue to expand the intellectual

property portfolio underlyin[redacted]ications filed worldwide that disclose and claim various aspects of the multifunctional degraders and methods for their use. Since entering into the license agreement with Yale in 2021, Yale has filed more than eight additional patent applications covering this technology.

About Biohaven.

Biohaven is a global clinical-stage biopharmaceutical company focused on the discovery, development and commercialization of life-changing therapies for people with debilitating neurological and neuropsychiatric diseases, including rare disorders. Biohaven is advancing a pipeline of best-in-class therapies for diseases with little or no treatment options, leveraging its proven drug development capabilities and proprietary platforms, including Kv7 ion channel modulation for epilepsy and neuronal hyperexcitability; glutamate modulation for obsessive-compulsive disorder and spinocerebellar ataxia and myostatin inhibition for neuromuscular diseases. Biohaven's portfolio of early- and late-stage product candidates also includes discovery research programs focused on TRPM3 channel activation for neuropathic pain and CD-38 antibody recruiting, bispecific molecules for multiple myeloma. More information about Biohaven is available at www.biohaven.com.

Forward-Looking Statements

This news release includes forward-looking statements within the meaning of the Private Securities Litigation Reform Act of 1995. The use of certain words, including "continue", "plan", "will", "believe", "may", "expect", "anticipate" and similar expressions, is intended to identify forward-looking statements. Investors are cautioned that any forward-looking statements, including statements regarding the future development, timing and potential marketing approval and commercialization of development candidates are not guarantees of future performance or results and involve substantial risks and uncertainties. Actual results, developments and events may differ materially from those in the forward-looking statements as a result of various factors including: Biohaven's ability to complete the offering of its common shares on the proposed terms, or at all; Biohaven's expectations related to the use of proceeds from the offering of its common shares; the expected timing, commencement and outcomes of Biohaven's planned and ongoing clinical trials; the timing of planned interactions and filings with the FDA; the timing and outcome of expected regulatory filings; complying with applicable U.S. regulatory requirements; the potential commercialization of Biohaven's product candidates; the potential for Biohaven's product candidates to be first in class or best

in class therapies; and the e[redacted]oduct candidates. Additional important factors to be considered in connection with forward- looking statements are described in Biohaven's filings with the Securities and Exchange Commission, including within the sections titled "Risk Factors" and "Management's Discussion and Analysis of Financial Condition and Results of Operations". The forward-looking statements are made as of the date of this new release, and Biohaven does not undertake any obligation to update any forward-looking statements, whether as a result of new information, future events or otherwise, except as required by law.

MoDEs is a trademark of Biohaven Therapeutics Ltd.

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SOURCE Biohaven Ltd.

EXHIBIT 36

US005985826A

United States Patent [19]

Theodore et al.

[11] **Patent Number: 5,985,826**

[45] **Date of Patent: Nov. 16, 1999**

[54] **METHODS OF USING HEPATIC-DIRECTED COMPOUNDS IN PRETARGETING STRATEGIES**

[75] Inventors: **Louis J. Theodore**, Lynnwood; **Donald B. Axworthy**; **John M. Reno**, both of Brier, all of Wash.

[73] Assignee: **NeoRx Corporation**, Seattle, Wash.

[21] Appl. No.: **08/808,024**

[22] Filed: **Mar. 3, 1997**

Related U.S. Application Data

[62] Division of application No. 08/351,651, Dec. 7, 1994.

[51] **Int. Cl.**[6] **A61K 31/70**; A61K 31/715; A61K 31/73; A61K 38/14

[52] **U.S. Cl.** **514/8**; 514/24; 514/42; 514/54

[58] **Field of Search** 424/1.69, 1.73, 424/85.1, 85.2, 85.4, 178.1, 179.1, 181.1; 514/2.8, 12.21, 24, 42, 44, 54

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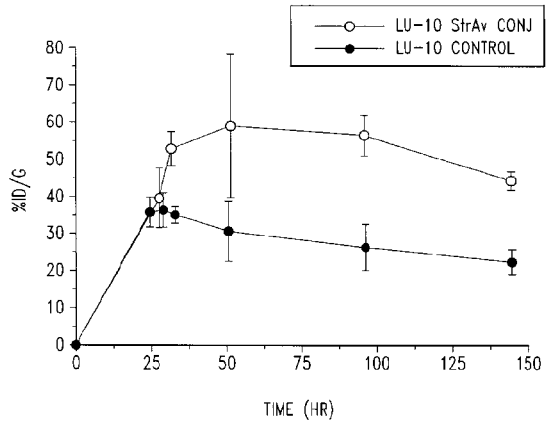
Primary Examiner—Jeffrey E. Russel
Attorney, Agent, or Firm—Seed and Berry LLP

[57] **ABSTRACT**

Hepatic-directed compounds, reagents useful in making such compounds and associated methods and compositions are disclosed. Hepatic-directed compounds are processed by metabolic mechanisms, which generally differ in degree or in kind from the metabolic mechanisms encountered by compounds which are not so directed. Hepatic-directed compounds useful in the methods disclosed include a hexose cluster characterized by multiple hexose residues connected in an iteratively branched configuration. In one embodiment, the hexose cluster comprises at least four hexose residues with each branch of the configuration having two prongs. In another embodiment, the hexose cluster comprises at least nine hexose residues with each branch of the configuration having three prongs.

5 Claims, 7 Drawing Sheets



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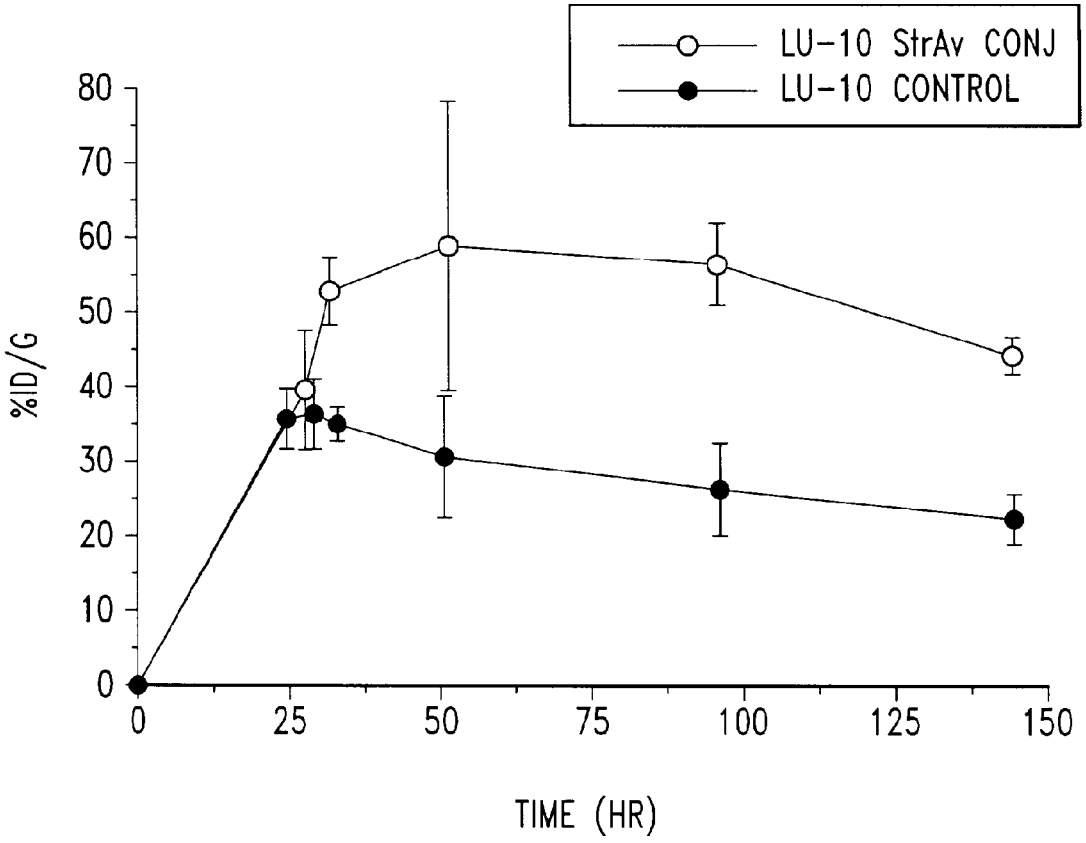
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LU-10 CONTROL
%ID/G
80
70
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50
40
30
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10
0
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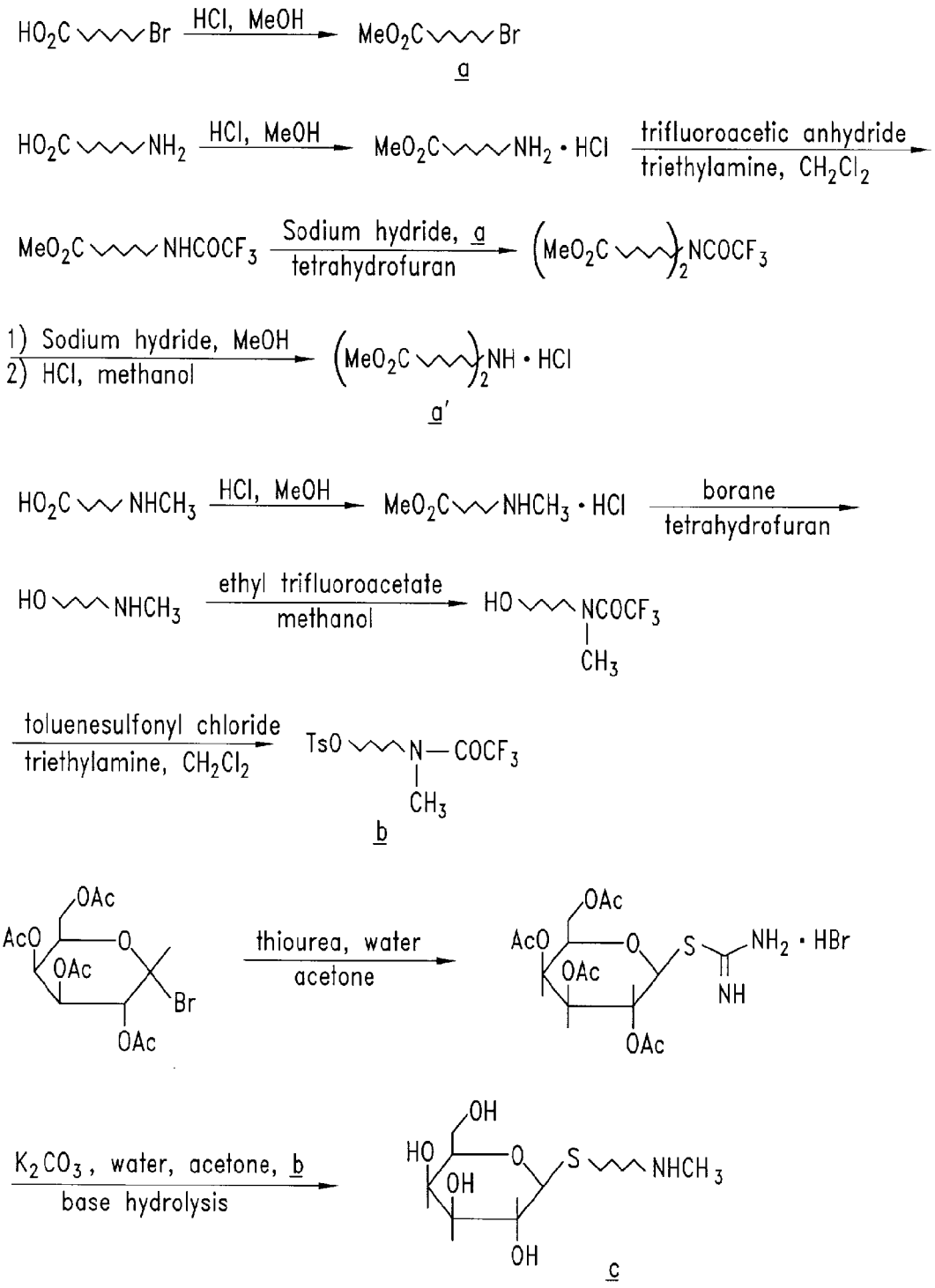
25
50
75
100
125
150
TIME (HR)

Fig. 1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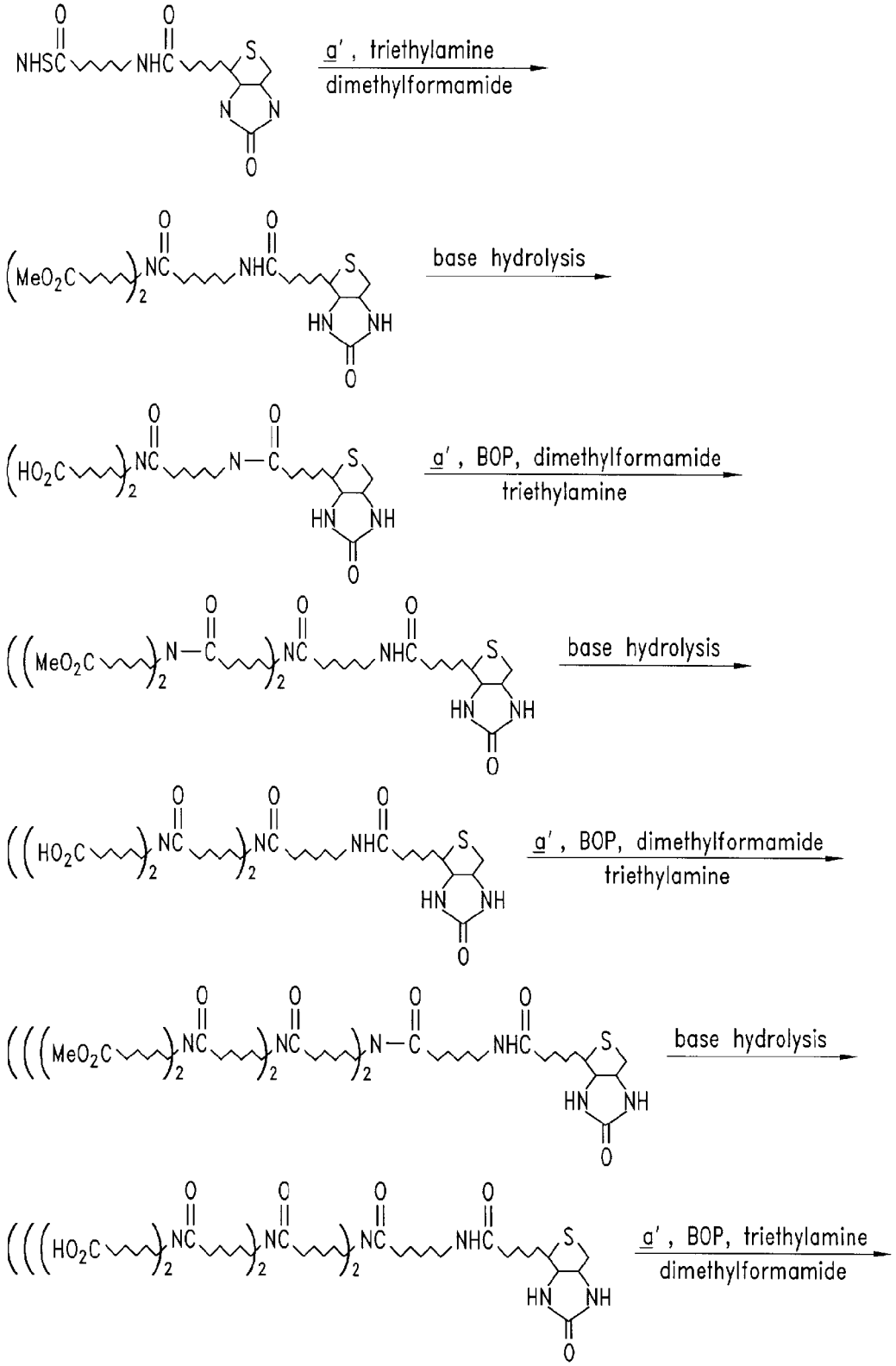
HCl, MeOH
MeO2C Br
a
HO2C NH2
HCl, MeOH
MeO2C NH2 · HCl
trifluoroacetic anhydride
triethylamine, CH2Cl2
MeO2C NHCOCF3
Sodium hydride, a
tetrahydrofuran
(MeO2C)2NCOCF3
1) Sodium hydride, MeOH
2) HCl, methanol
(MeO2C)2NH · HCl
a'
HO2C NHCH3
HCl, MeOH
MeO2C NHCH3 · HCl
borane
tetrahydrofuran
HO NHCH3
ethyl trifluoroacetate
methanol
HO NCOCF3 CH3
toluenesulfonyl chloride
triethylamine, CH2Cl2
TsO N COCF3 CH3
b
AcO OAc O Br OAc
thiourea, water
acetone
AcO OAc O S NH2 · HBr NH OAc
K2CO3, water, acetone, b
base hydrolysis
HO OH O S NHCH3 OH OH
c

Fig. 2A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dimethylformamide
base hydrolysis
a', BOP, dimethylformamide
triethylamine
base hydrolysis
a', BOP, dimethylformamide
triethylamine
base hydrolysis
a', BOP, triethylamine
dimethylformamide

Fig. 2B

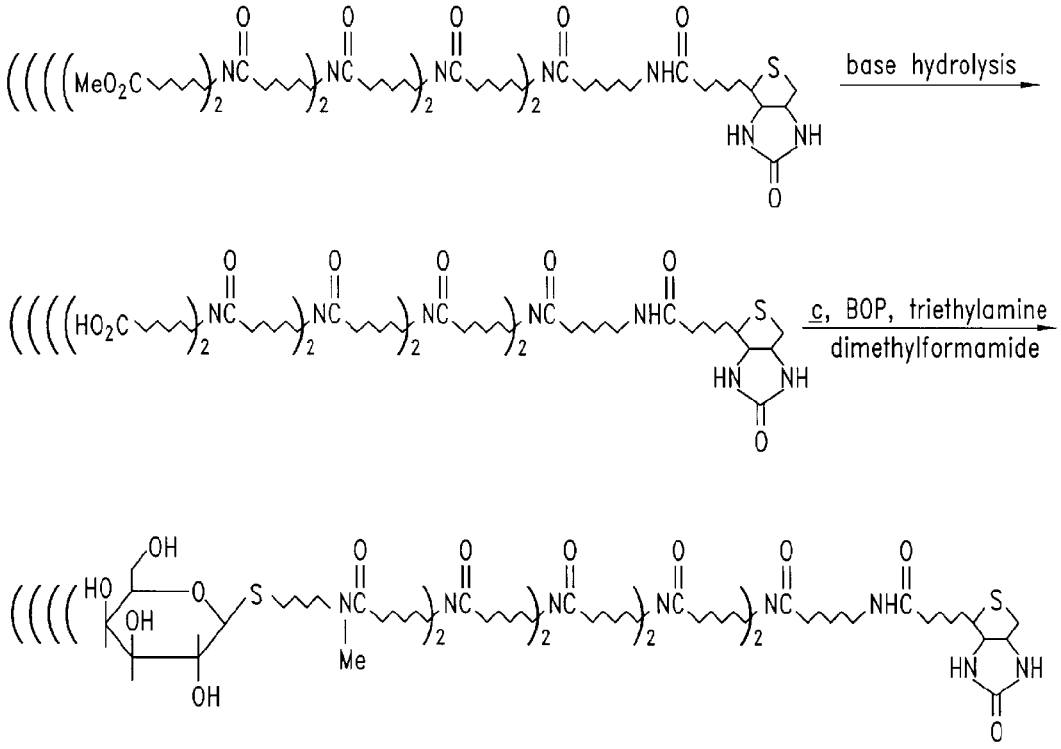
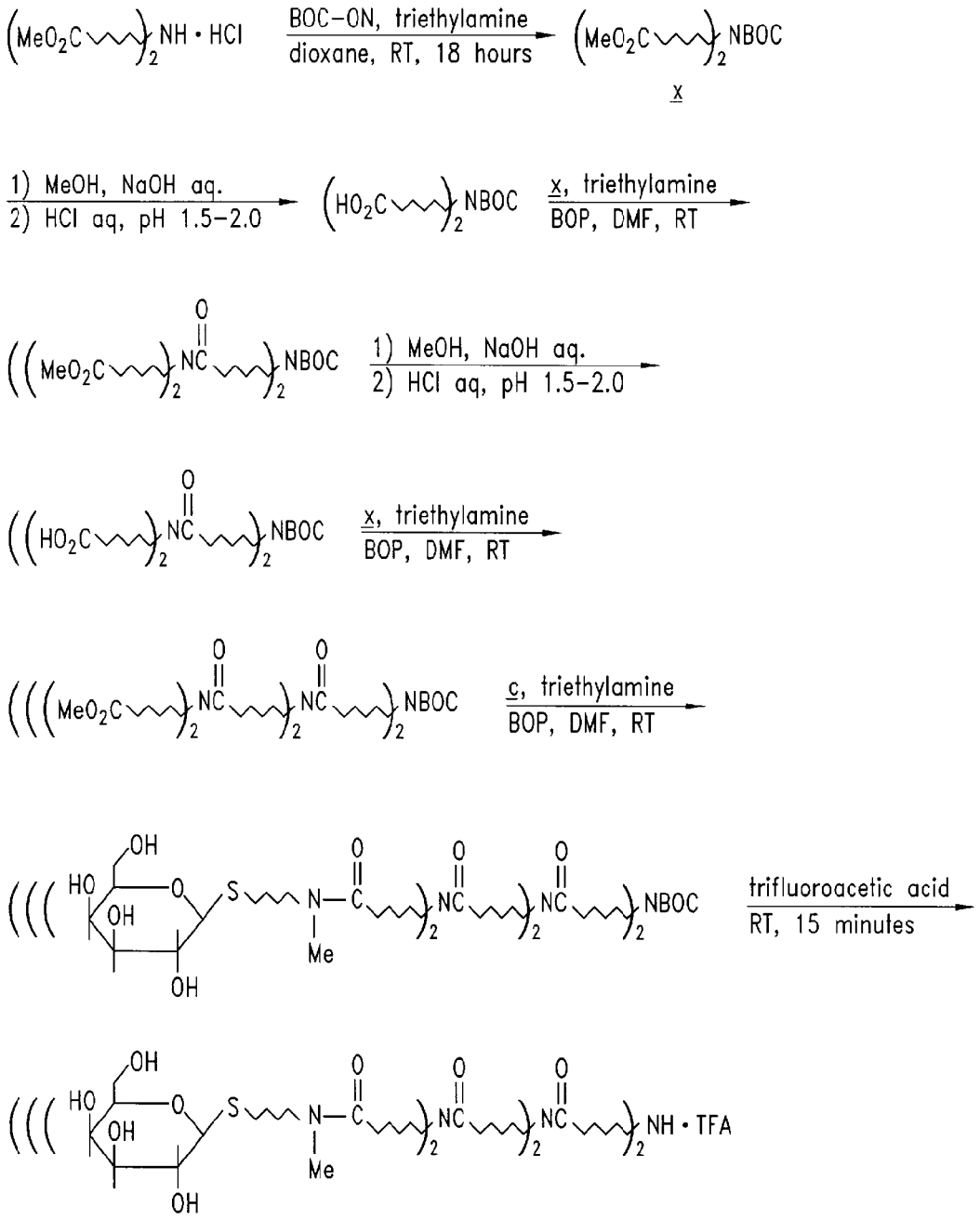


Fig. 2C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dioxane, RT, 18 hours
(MeO2C)2NBOC
x
1) MeOH, NaOH aq.
2) HCl aq, pH 1.5–2.0
(HO2C)2NBOC
x, triethylamine
BOP, DMF, RT
((MeO2C)2NC)2NBOC
1) MeOH, NaOH aq.
2) HCl aq, pH 1.5–2.0
((HO2C)2NC)2NBOC
x, triethylamine
BOP, DMF, RT
(((MeO2C)2NC)2NC)2NBOC
c, triethylamine
BOP, DMF, RT
trifluoroacetic acid
RT, 15 minutes
NH • TFA

Fig. 3

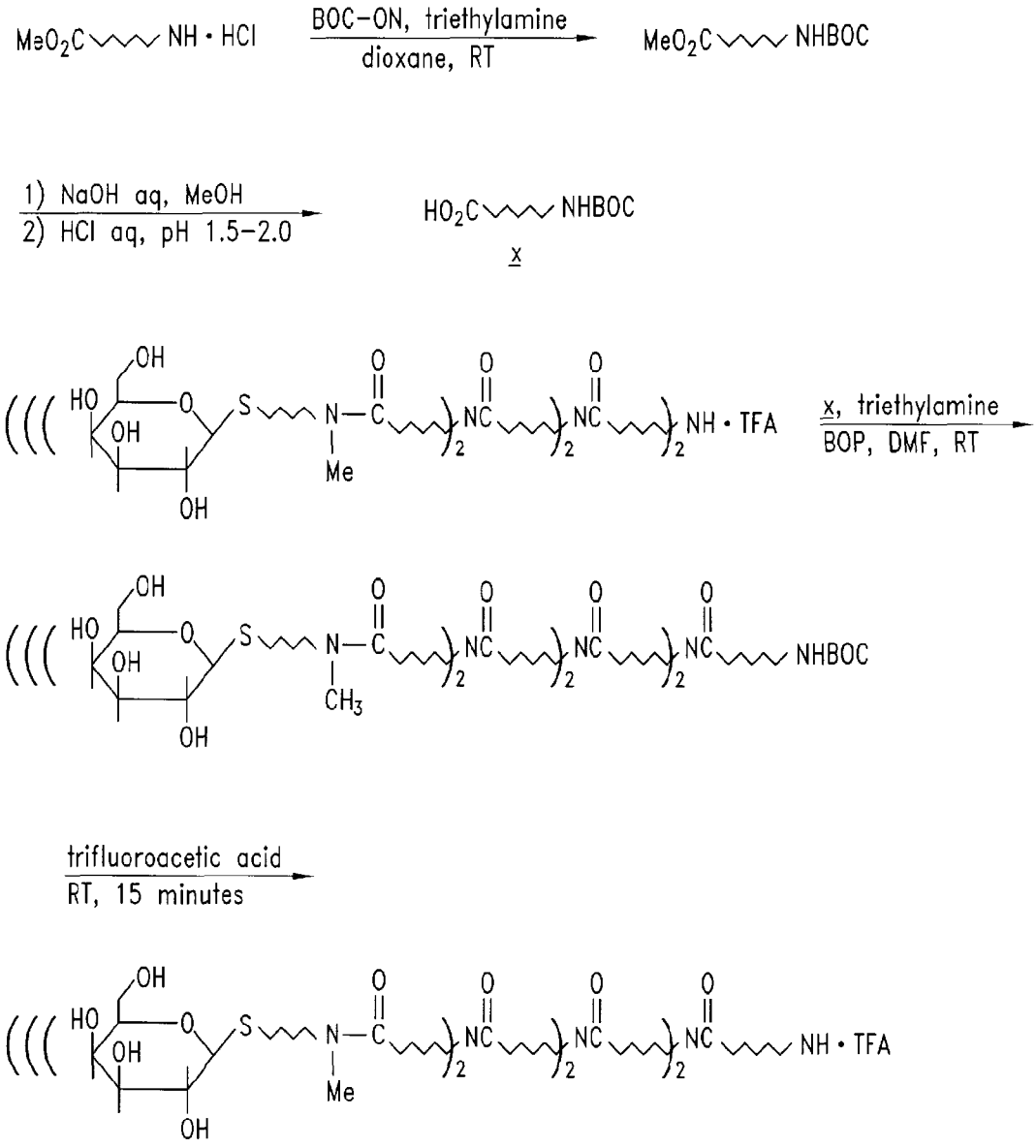

MeO2C NH • HCl
BOC–ON, triethylamine
dioxane, RT
MeO2C NHBOC
1) NaOH aq, MeOH
2) HCl aq, pH 1.5–2.0
HO2C NHBOC
x
x, triethylamine
BOP, DMF, RT
NH • TFA
NHBOC
trifluoroacetic acid
RT, 15 minutes
NH • TFA

Fig. 4

N, N′ – Bis (2–disulfidyl–4–methylphenyl) – γ,γ′ – diamino isovalerate N–hydroxysuccinimide

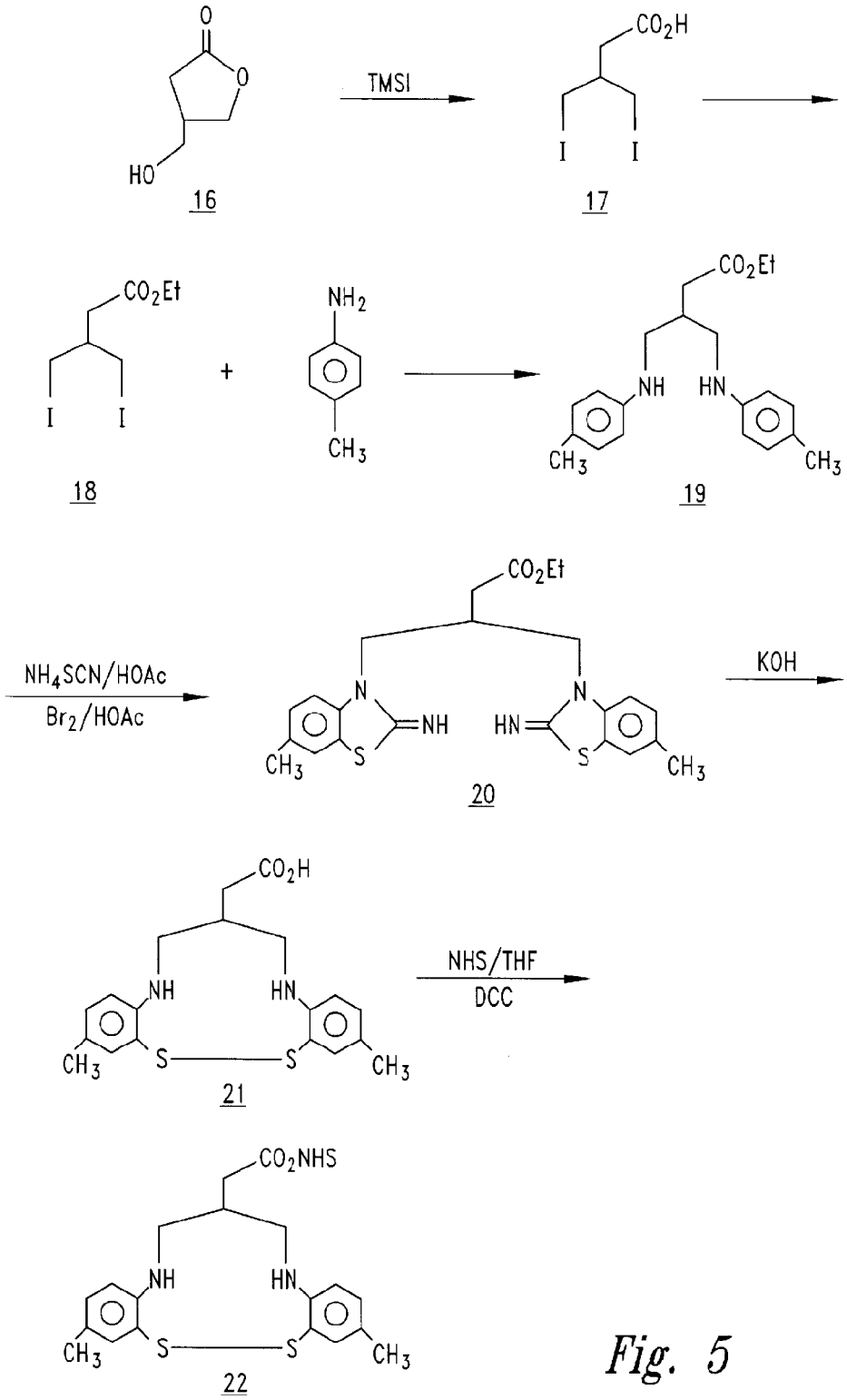


Fig. 5

METHODS OF USING HEPATIC-DIRECTED COMPOUNDS IN PRETARGETING STRATEGIES

This application is a divisional of U.S. application Scr. No. 08/351,651, filed Dec. 7, 1994.

TECHNICAL FIELD

The present invention relates to hepatic-directed compounds, reagents useful in making such compounds and associated methods and compositions. Hepatic-directed compounds are processed by metabolic mechanisms, which generally differ in degree or in kind from the metabolic mechanisms encountered by compounds which are not so directed. Hepatic-directed compounds are eliminated from the recipient via the liver and, generally, exhibit a decreased serum half-life in comparison to non-directed counterpart compounds.

BACKGROUND OF THE INVENTION

Conventional cancer therapy is plagued by the problem that the generally attainable targeting ratio (ratio of administered dose localizing to tumor versus administered dose circulating in blood or ratio of administered dose localizing to tumor versus administered dose migrating to bone marrow) is low. Improvement in targeting ratio dose to tumor is sought.

One method employed in efforts to improve targeting ratio is to decrease the serum concentration of a compound. One method of decreasing the serum concentration of an administered compound is to subsequently administer a molecule designed to be eliminated rapidly via the liver and to bind to the first administered compound. Galactose-HSA-biotin conjugates, discussed in PCT patent application No. PCT/US93/05406 facilitate elimination of circulating targeting agent-streptavidin conjugates from the bloodstream. Galactosylated antibodies directed to a portion of a previously administered molecule have also been employed for this purpose.

In addition, the liver is susceptible to a variety of conditions for which liver delivery of an active agent would be useful. In these circumstances, delivery of active agent via the hepatic artery has been proposed. This methodology is invasive and, therefore, other methods of active agent delivery to the liver are sought.

SUMMARY OF THE INVENTION

The present invention is directed to hepatic-directed compounds, reagents and methods for the preparation and use of such compounds. Hepatic-directed compounds may be employed to deliver an active agent to the liver, to improve targeting ratio, or both. Hepatic directed compounds may also be employed to direct previously administered moieties or toxic or potentially toxic moieties to a liver metabolic pathway for elimination.

One embodiment of hepatic-directed compounds of the present invention generally includes a director moiety and an active agent. In this embodiment of the present invention, one or more active agents may be directly or indirectly bound to the director moiety. Examples of indirect binding include the use of polymeric carriers, liposomes, particulate dosage forms and the like. Such hepatic-directed compounds are especially useful for delivery of active agents to the liver to address liver conditions. The director moiety directs localization of the hepatic-directed compound to the liver, and the active agent addresses the ailment.

In the situation wherein an improvement in targeting ratio is sought, hepatic-directed compounds of the present invention generally include a targeting moiety directed to the target cell population to be treated/diagnosed as well as a director and, optionally, an active agent-type effector. Under these circumstances, the targeting moiety directs the localization of the compound to target cells, the active agent addresses the ailment, and the director moiety facilitates removal of the compound from circulation via the liver thereby reducing exposure of the recipient's normal tissues to the active agent.

In another embodiment of the present invention, the hepatic-directed compounds of the present invention generally include a director moiety and a binding moiety, which recognizes a previously administered agent or other toxic agent in the bloodstream or extravascular fluid space of the recipient. Such hepatic-directed compounds are especially useful for clearance of previously administered molecules, such as targeting moiety-receptor constructs designed to accrete to target sites and facilitate localization of subsequently administered active agent-containing molecules that recognize the receptors. Consequently, this embodiment of the present invention is particularly amenable to use in pretargeting protocols as described herein.

Alternatively, the binding moiety may be directed to toxic or potentially toxic moieties located in the recipient's circulation or extravascular fluid space. The director moiety directs localization of the hepatic-directed compound to the liver, and the binding moiety binds to the molecule to be eliminated via the hepatic pathway.

Preferred director moieties of the present invention are branched sugar constructs (i.e, sugar clusters) that are recognized by a population of receptors on the liver. Exemplary sugars for this purpose are galactose and mannose. The branched configuration typically facilitates recognition of the sugars by liver receptors, as such receptors often most efficiently process clusters of sugars of certain configurations.

More preferred director moieties according to the present invention contain galactose or galactose derivatives. An embodiment of such preferred director moieties incorporates a multiple of 4 galactoses. Director moieties having 4, 8, 16 and 32 galactose residues are generally preferred for use in pretargeting aspects of the present invention. Alternative branching structures, such as those having 3, 9, 27, etc. sugars are also contemplated by the present invention.

Director moieties may be incorporated into hepatic-directed compounds using appropriate reagents therefor. Director reagents of the present invention incorporate a galactose cluster, such as those described above, and a functional group, such as an amine active ester, maleimide, alkyl halide, hydrazide, thiol, imidate, aldehyde or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates the tumor uptake profile of NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody.

FIGS. **2***a*, **2***b* and **2***c* schematically depict the preparation of a sixteen galactose cluster-biotin conjugate.

FIG. **3** schematically depicts the synthesis of an eight galactose-containing galactose cluster.

FIG. **4** schematically shows the synthesis of an extended eight galactose-containing galactose cluster.

FIG. **5** schematically shows the synthesis of N,N'-bis (disulfidyl-4-methylphenyl) -gamma, gamma'-diamino-isovalerate N-hydroxysuccinimide.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

Targeting moiety: A molecule that binds to a defined population of cells. The targeting moiety may bind a receptor, an oligonucleotide, an enzymatic substrate, an antigenic determinant, or other binding site present on or in the target cell population. Antibody is used throughout the specification as a prototypical example of a targeting moiety. Tumor is used as a prototypical example of a target in describing the present invention.

Licand/anti-licand pair: A complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include zinc finger protein/dsDNA fragment, enzyme/inhibitor, hapten/antibody, lectin/carbohydrate, ligand/receptor, S-protein/S-peptide, head activator protein (which binds to itself), cystatin-C/cathepsin B, and biotin/avidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

Anti-ligand: As defined herein, an "anti-ligand" demonstrates high affinity, and preferably, multivalent binding of the complementary ligand. Preferably, the anti-ligand is large enough to avoid rapid renal clearance, and contains sufficient multivalency to accomplish crosslinking and aggregation of targeting moiety-ligand conjugates. Univalent anti-ligands are also contemplated by the present invention. Anti-ligands of the present invention may exhibit or be derivatized to exhibit structural features that direct the uptake thereof, e.g., galactose residues that direct liver uptake. Avidin and streptavidin are used herein as prototypical anti-ligands.

Avidin: As defined herein, "avidin" includes avidin, streptavidin and derivatives and analogs thereof that are capable of high affinity, multivalent or univalent binding of biotin.

Ligand: As defined herein, a "ligand" is a relatively small, soluble molecule that binds with high affinity by anti-ligand and preferably exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human. Biotin is used as the prototypical ligand.

Lower Affinity Ligand or Lower Affinity Anti-Liqand: A ligand or anti-ligand that binds to its complementary ligand-anti-ligand pair member with an affinity that is less than the affinity with which native ligand or anti-ligand binds the complementary member. Preferably, lower affinity ligands and anti-ligands exhibit between from about 10^{-6} to 10^{-10}M binding affinity for the native form of the complementary anti-ligand or ligand. For avidin/streptavidin and other extremely high affinity binding molecules, however, lower affinity may range between 10^{-6} to 10^{-13}M. Lower affinity ligands and anti-ligands may be employed in clearing agents or in active agent-containing conjugates of the present invention.

Active Agent: A diagnostic or therapeutic agent ("the payload"), including radionuclides, drugs, anti-tumor agents, toxins and the like. Radionuclide therapeutic agents are used as prototypical active agents.

N_xS_y Chelates: As defined herein, the term "N_xS_y chelates" includes bifunctional chelators that are capable of (i) coordinately binding a metal or radiometal and (ii) covalently attaching to a targeting moiety, ligand or anti-ligand. Particularly preferred N_xS_y chelates have N_2S_2 and N_3S cores. Exemplary N_xS_y chelates are described in Fritzberg et al., *Proc. Natl. Acad. Sci. USA* 85:4024–29, 1988; in Weber et al., *Bioconi. Chem.* 1:431–37, 1990; and in the references cited therein, for instance.

Pretargeting: As defined herein, pretargeting involves target site localization of a targeting moiety that is conjugated with one member of a ligand/anti-ligand pair; after a time period sufficient for optimal target-to-non-target accumulation of this targeting moiety conjugate, active agent conjugated to the opposite member of the ligand/anti-ligand pair is administered and is bound (directly or indirectly) to the targeting moiety conjugate at the target site (two-step pretargeting). Three-step and other related methods described herein are also encompassed.

Clearing Agent: An agent capable of binding, complexing or otherwise associating with an administered moiety (e.g., targeting moiety-ligand, targeting moiety-anti-ligand or anti-ligand alone) present in the recipient's circulation, thereby facilitating circulating moiety clearance from the recipient's body, removal from blood circulation, or inactivation thereof in circulation. The clearing agent is preferably characterized by physical properties, such as size, charge, configuration or a combination thereof, that limit clearing agent access to the population of target cells recognized by a targeting moiety used in the same treatment protocol as the clearing agent.

Conjugate: A conjugate encompasses chemical conjugates (covalently or non-covalently bound), fusion proteins and the like.

Hepatic-directedected compounds: Conjugates generally including a director and an effector. One or more effector molecules may be directly or indirectly bound to one or more directors.

Indirect Binding: Binding of effector molecule(s) to director molecule(s) via a carrier, such as a polymer, a liposome, a particulate dosage form or the like.

Direct Binding: Direct chemical linkage between components of a hepatic-directed compound or such chemical linkages incorporating a spacer, extender, or other chemical linker molecule designed as a linker rather than as a carrier.

Director: A moiety capable of directing the clearance of a component to which it is bound upon administration or of a component to which it becomes bound in vivo. Director moieties of the present invention direct clearance via the hepatic pathway.

Effector: A moiety capable of achieving a desired effect for a specific application, such as an active agent; a binding moiety including a ligand, an anti-ligand or the like; a targeting moiety; or the like.

Binding Moiety: A ligand, anti-ligand or other moiety capable of in vivo association with a previously administered molecule (bearing the complementary ligand or anti-ligand, for example) or with another toxic or potentially toxic molecule present in the recipient's circulation or extravascular fluid space via recognition by the binding moiety of an epitope associated with the toxic or potentially toxic molecule.

Sucar cluster: A director moiety having a plurality of sugar residues configured to be recognized by a liver receptor. Such clusters are preferably constructed of sugar residues connected in a branched configuration, and are attached to other components of a sugar cluster-containing conjugate via a single point of attachment. Preferably, the branching network consists of two or three pronged branches, i.e.,

consists of 2, 4, 8, 16, 32 or 64 sugar residues or consists of 3, 9, 27, or 81 sugar residues.

Sugar Cluster Clearing Acent: A hepatic directed compound designed for use as a clearing agent in a pretargeting protocol incorporating a sugar cluster director.

Galactose cluster: A director moiety having from about 3 to about 100 galactose residues connected in a branched configuration, with constructs involving less than 50 galactose residues preferred. Preferably, the branching network consists of two or three pronged branches, i.e., consists of 2, 4, 8, 16, 32 or 64 galactose residues or consists of 3, 9, 27, or 81 galactose residues.

Galactose Cluster Clearing Agent: A hepatic directed compound designed for use as a clearing agent in a pretargeting protocol incorporating a galactose cluster director.

Director Reagent: A reagent comprising a directing portion and one or more functional groups for binding to an effecting portion to form a hepatic-directed compound.

An embodiment of the present invention is directed to hepatic-directed compounds suitable for delivery of active agent effectors to liver targets, which hepatic-directed compounds include:

a director including a cluster of sugar residues which is capable of directing liver uptake of the compound; and

an active agent directly or indirectly bound to the director capable of diagnostic or therapeutic application with respect to a liver ailment.

In this embodiment of the present invention, the director serves to deliver the active agent to the liver target. The active agent provides a diagnostic or therapeutic benefit at the liver target. Further, an optional active agent carrier facilitates delivery of a plurality of active agent molecules or multiple active agents to the liver target.

Another embodiment of the present invention is directed to hepatic-directed compounds suitable for reduction of background active agent or targeting moiety concentration in the circulation or extravascular fluid space of a recipient, which hepatic-directed compounds include:

a director including a cluster of sugar residues which is capable of directing liver uptake of the compound;

a targeting moiety which localizes to a target of interest, which targeting moiety optionally is covalently or non-covalently bound to a receptor; and, optionally,

an active agent, directly or indirectly bound to the director or to the targeting moiety (preferably to the targeting moiety), capable of diagnostic or therapeutic application with respect to the target.

In this embodiment of the present invention, the targeting moiety localizes to target, either delivering a receptor or an active agent thereto. The director promotes elimination of the hepatic-directed moiety via the liver to reduce non-target accumulation of the hepatic-directed molecule. The optional receptor, if employed, provides a binding site for a subsequently administered active agent-containing construct. The optional active agent, if employed, provides a diagnostic or therapeutic benefit at the target. Further, an optional active agent carrier facilitates delivery of a plurality of active agent molecules or multiple active agents to the target.

Further embodiments of the present invention are directed to hepatic-directed compounds suitable for directing the metabolic pathway for elimination of molecules present in the circulation or extravascular fluid space of a recipient, which hepatic-directed compounds include:

a director including a cluster of sugar residues which is capable of directing liver uptake the compound; and

a binding moiety directly or indirectly bound to the director capable of in vivo complexation with certain molecules present in the circulation or extravascular fluid space of the recipient.

In this embodiment of the present invention, the director serves to direct the biodistribution of the hepatic-directed molecule and the constructs with which it becomes associated in vivo. The binding moiety facilitates in vivo association with previously administered compounds or with toxic or potentially toxic moieties resident in the circulation or extravascular fluid space of the recipient. Further, an optional binding agent carrier facilitates transport of a plurality of binding agent molecules in the circulation or extravascular fluid space.

When radionuclides are employed as active agents, constructs of the present invention include: hepatic directed compounds incorporating chelates for subsequent complexation of radionuclide therein (conjugation via the post-formed approach) as well as hepatic-directed compounds incorporating radionuclides previously complexed with radionuclide (conjugation via the pre-formed approach).

Hexose clusters are preferably employed as directors in the practice of the present invention. Galactose clusters are the prototypical hexose clusters employed for the purposes of this description. Design of hexose clusters of the present invention is conducted with the following criteria in mind, as set forth in the context of the design of a galactose cluster:

1) Number of Galactoses in a Cluster;

2) Distance Between Galactoses in the Cluster; and

3) Distance Between Galactose Cluster and a Conjugate Component Which Must Bind to Circulating Molecules or to Target.

With regard to criterion number 1, literature indicates that galactose receptors on the surface of human hepatocytes are grouped as heterotrimers and, perhaps, bis-heterotrimers. See, for example, Hardy et al., *Biochemistry*, 24: 22–8, 1985. For optimal affinity to such receptors, the present inventors believe that each galactose cluster should preferably contain at least three galactose residues. In general, the greater the number of sugars in a cluster, the greater the propensity for the cluster to be recognized by liver receptors.

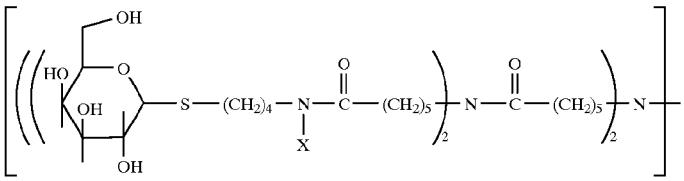
Increased sugar cluster size may impair binding to circulating molecules or to target. If significant impairment in such binding (e.g. reduction to <20% of native targeting moiety or binding moiety binding capability) is observed, a longer linker should be employed between the two moieties or such large clusters should not be used in hepatic-directed compounds of the present invention. The present invention embraces hexose clusters with any number of hexose residues or any mixture thereof which results in efficacious liver clearance of the resultant hepatic-directed molecule.

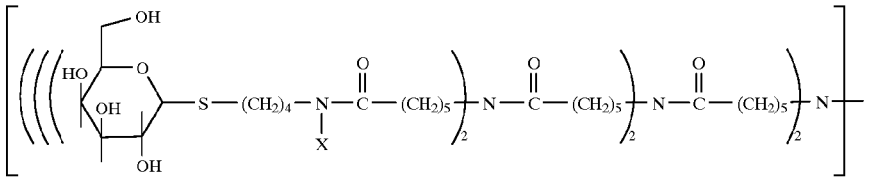
With respect to criterion number 2, the galactose receptors within each trimer are separated from each other by distances of 15, 22 and 25 angstroms. Consequently, the present inventors believe that the galactoses within a cluster should preferably be separated by flexible linkers allowing separation of at least 25 angstroms. The spacing between sugar residues is likely to be more important if the number of sugar residues is small. With larger constructs, appropriate spacing is likely to occur with respect to sugar residues that are not immediate neighbors (i.e., sugar residues that are farther apart than those that are immediate neighbor). Assuming an average bond length of 1.5 angstroms, preferred sugar clusters of the present invention are characterized by separation of neighboring sugar residues by about 10 bond lengths or more. Other preferred constructs involve galactose clusters characterized by separation of neighboring sugar residues by about 25 bond lengths or more.

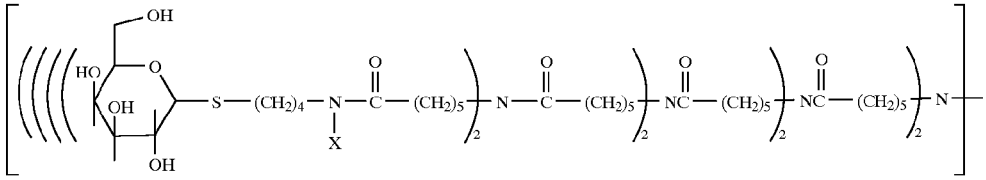
Regarding criterion number 3, the distance between the targeting moiety and/or the binding moiety component and the galactose cluster should be sufficient to obviate any adverse steric effects upon binding capability of those components caused by the size or orientation of the galactose cluster. This distance is preferably greater than about 7 bond lengths or about 10 angstroms. If necessary, an extender molecule is incorporated between the relevant conjugate components to provide the requisite distance. For example, such extenders may be positioned between the galactose cluster and a linker (which joins the galactose cluster and the targeting or bindinding component) or between the targeting or binding component and the linker to provide the requisite distance.

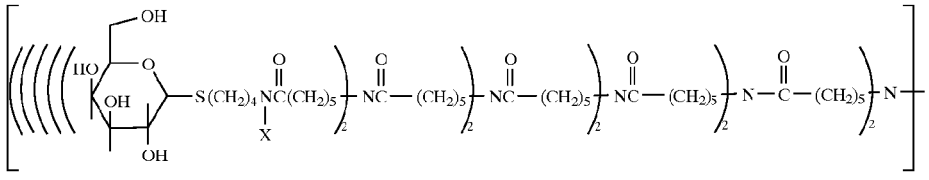
While the foregoing parameters appear to be optimal for galactose, it should be noted that these factors may vary with other hexoses or mixtures thereof, which may or may not bind to the same receptors, or may bind differently. Given the teachings in this application, one skilled in the art can, using available synthesis techniques, prepare constructs incorporating other hexose clusters and identify those constructs which provide optimal performance.

Any branched sugar structures that meet the criteria described above may be employed in the practice of the present invention. Preferred galactose clusters of the present invention are of the following structures:





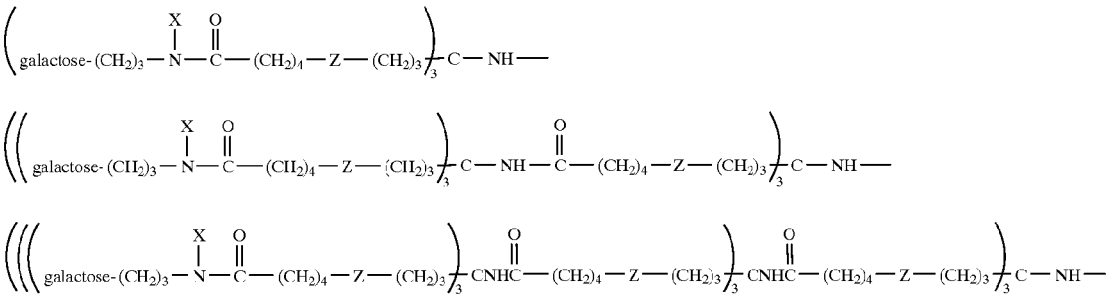




wherein X is preferably H or methyl, resulting in galactose clusters bearing 4, 8, 16 and 32 galactose residues, respectively. Further iteration in the branching scheme allows expansion of the galactose cluster to include 32, 64, etc. galactose residues. In addition, the linker moiety between the sugar itself and the branching structure (shown as —S—$(CH_2)_4$—NX—) may be variable in length.

Alternative branching structures may also be employed in the design of galactose clusters in accordance with the present invention. For example, other constructs wherein the branching results in a doubling of the number of galactose residues may be employed. In addition, constructs wherein branching results in a tripling or other convenient multiplying of the number of galactose residues are also contemplated by the present invention.

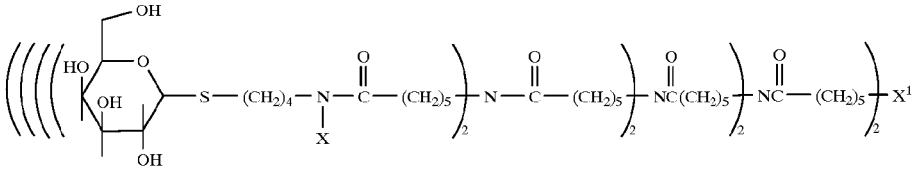
Another potential branching construction is based upon the molecule bis-homotris: $(HO—CH_2)_3—C—NH_2$. The sulfhydryl-containing derivative of this molecule may also be used. In this embodiment of the present invention, each arm of the bis-homotris molecule is extended and terminated in a carboxylic acid: $(HO_2C—(CH_2)_y—Z—(CH_2)_3—C—NH_2$, where Z is S or O and y ranges from 1 to about 10. For this embodiment of the present invention, a preferred galactose cluster is characterized by the following structures:



wherein X is preferably H or methyl; y ranges from 1 to about 10; and Z is O or S. The above structures bear 3, 9 and 27 galactose residues, respectively. Further iteration of the branching allows expansion to include 81, etc. galactose residues.

Also, X may be a lower alkyl moiety different from methyl, such as ethyl, t-butyl and the like. X may also be a lower alkyl group bearing a heteroatom, such as a lower alkyl acid, ester, aldehyde, ketone or ether. The purpose of X is to provide steric inhibition to metabolic/catabolic enzymes that may cleave the amide bond. X should not alter the function of the agent to which it is attached and, therefore, may be altered to increase/decrease solubility, charge or other physical property as necessary for a given application.

Galactose cluster director molecules are incorporated into hepatic-directed compounds using director reagents. A family of director reagents having different functional groups can be employed for binding of such director reagents to various other molecules to form a variety of hepatic-directed compounds in accordance with the present invention. A preferred family of director reagents of the present invention may be represented by the following formula:



wherein X' bears an available functional group and X is H or methyl, resulting in galactose clusters bearing 16 galactose residues. Other related director reagents bearing an alternative multiple of 4 galactose residues are analogously structured. The available functional group of X' is selected in accordance with the nature of the other component(s) of the hepatic-directed compound. Examples of useful X' functional groups include amines, activated esters, maleimides, isocyanates, isothiocyanates alkyl halide (e.g., iodoacetate), alpha-halo-ketones, alpha-halo-acids, hydrazides, thiols, imidates, aldehydes, photolytic conjugating groups, and the like.

For example, activated esters may be employed to conjugate the galactose cluster to amines (primary or secondary), hydroxyls, sulfhydryls, and the like. Maleimides facilitate conjugation to thiols and the like. Isocyanates and isothiocyanates may be employed for conjugation to amines and the like. Alkyl halides are useful for conjugation to thiols, hydroxyls, amines and the like. Hydrazide groups facilitate conjugation to activated esters, aldehydes, ketones and the like. Thiols may be employed to conjugate the galactose cluster to thiols, maleimides, alkyl halides, alpha-halo-ketones, and the like. Imidates facilitate conjugation to amines the like. Aldehydes may be employed for conjugation to amines, via Schiff base formation with or without reduction, and the like.

These same X' functional groups can be employed in director reagents for any director reagent family of the present invention. A family of director reagents is formed from a single molecule structured as follows: Hexose cluster-base (in the structural sense) functionality-available (in the steric sense) functionality, wherein the base functionality is amenable to derivatization to provide X' moieties bearing available functional groups that are the same or different from the base functionality. Examples of base functionalities are —NH$_2$, active ester, maleimide, sulfhydryl, and the like. For an —NH$_2$ base functionality, appropriate X' groups include the following:

amine;

—NR—CO—(CH$_2$)$_2$—O—NHS;

—NR—CO—(CH$_2$)$_2$—O—NHS—SO$_3$Na;

—NR—CO—(CH$_2$)$_2$—O—tetrafluorophenyl;

—maleimide;

—NR-extender-maleimide, wherein the extender is an aminocaproate group, —(CH$_2$)$_n$ or the like, wherein n ranges from 1 to about 10;

—3(2-pyridyldithio)propionamide;

—NR—CO—CH$_2$—SH;

—NR—CO—CH$_2$—halide, preferably I or Br;

—NR—CO—NH—NH$_2$;

—NR—CO—(CH$_2$)$_n$—CO—NH—NH$_2$, wherein n ranges from 1 to about 10;

—NR—CO—(CH$_2$)$_2$C═NH$_2$+—OCH$_3$;

—NR—CO—CH$_2$—p—N$_3$—phenyl;

and the like.

In addition, the amine base functionality may additionally be N-alkylated to enhance stability against metabolic degrada-

tion or retention within hepatocytes. Consequently, R may be H, CH_3, CH_2COOH or the like.

Example VII indicates a method for preparation of a related member of the above-identified director reagent family (wherein X' is an amine and the galactose cluster incorporates 8 galactose residues). In addition, preparation of an embodiment of hepatic-directed compounds from such galactose cluster director reagents is described in Examples VIII and IX.

Hepatic-directededected molecules of the present invention may be formed using suitable linkers. Two component hepatic-directed molecules are formed using a bifunctional linker. For three component hepatic-directed compounds wherein none of the components are characterized by greater than one functional group suitable and available for conjugation to other components, trifunctional linkers are preferred. For three component hepatic-directed molecules wherein at least one of the components has two or more functional groups available and suitable for conjugation with other components, two bifunctional linkers are preferred for conjugate formation. Any linker or linker combination useful for linking the hepatic-directed compound component may be employed. Suitable trifunctional and bifunctional linkers are set forth below.

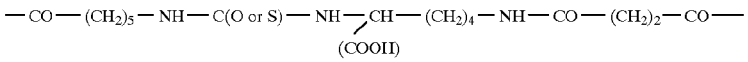
Functional groups that are "available" for conjugation are those that are not prevented by steric constraints from conjugate formation. Functional groups that are "nsuitable" for conjugation are those that are capable, in a chemical sense, of reacting with available functional groups associated with other conjugate components. In addition, conjugation of "suitable" functional groups does not substantially impair a necessary function of the component with which the functional group is associated. For example, a functional group located in the complementarity determining region of an antibody targeting moiety will generally not be "suitable" for conjugation, because the targeting ability of the antibody is likely to be substantially impaired by such binding.

Targeting moiety, binding moiety or active agent, and galactose cluster components of a three component hepatic-directed compound can be joined via a trifunctional linker, provided one of such components has the characteristics discussed above. Suitable trifunctional linkers are amenable to binding with functional groups available on the three conjugate components or any extender moieties employed in conjugate construction. A useful trifunctional linker is lysine, wherein the alpha-amino, epsilon-amino and carboxyl functional groups are used. One skilled in the art is capable of identifying other trifunctional linkers as well as of using such linkers as set forth herein.

Extender molecules useful in the present invention are bifunctional moieties capable of binding with either a targeting component, for example, and the linker or the galactose cluster component and the linker. Suitable extender molecules include aminocaproate moieties, IIS—$(CH_2)_n$COOH or an activated ester form thereof wherein n ranges from 2 to about 5, 4-aminobutanethiol, and the like. One of ordinary skill in the art is capable of identifying and using other suitable extender molecules as described herein. Alternatively, the extender function can be served by an appropriately constructed linker.

Also, binding facilitation moieties may also be employed in the present invention. Such moieties are bifunctional and facilitate binding the conjugate components, e.g., galactose cluster, targeting moiety, binding moiety, active agent, chelate, linker, and extender. Examples of such binding facilitation moieties include urea functionalities, thiourea functionalities, succinate bridges, maleimides and the like. Such binding facilitation moieties are amenable to identification and use by those skilled in the art.

An example of a linker-extender-binder facilitation system is shown below:



wherein the alpha-amine of the lysine linker is bound via a urea or thiourea functionality to an amino caproate spacer (which, in turn, binds to a galactose cluster that is not shown); the lysine carboxylate is available for linkage to a chelate (not shown); and the epsilon-amine of the lysine linker-is available for linkage to a lysine residue of the targeting component, for example, (not shown) via a succinate bridge. Other amino acid residues of the targeting component component, such as cysteine, may also be employed for binding purposes. Alternatively a maleimide—S—$(CH_2)_n$CO—binding facilitation moiety-extender combination may be employed to link the sugar residue with the lysine.

Alternatively, the galactose cluster may be linked to the chelate component which, in turn, is linked to the targeting component of the conjugate, for example, via two or more bifunctional linkers. Preferably, the targeting component, for example of the conjugate is attached last in the formation of a galactose cluster-containing conjugate. Suitable bifunctional linkers, such as bis-N,N-(6-(1-hydroxycarbonylhexyl) amine and the like, and linking methodologies can be identified and employed by one skilled in the art.

Preferably, the hepatic-directed compounds of the present invention designed for targeting to locations in the extravascular fluid space or for clearing molecules present in the extravascular fluid space are of a low enough molecular weight to provide for efficient diffusion into the extravascular fluid space. Molecular weights for such entities will preferably range from about 1500 to about 20,000 daltons.

When employing a radionuclide active agent, preparation of the hepatic-directed compound components via chemical methods can occur either prior to (post-formed approach) or following (pre-formed approach) complexation of the radionuclide within the chelate. Such conjugation is preferably conducted following radiometal complexation, however, unless the chelate employed in the conjugate is capable of binding the radionuclide rapidly at room temperature.

The "targeting moiety" of the present invention binds to a defined target cell population, such as tumor cells. Preferred targeting moieties useful in this regard include antibody and antibody fragments, peptides, and hormones. Proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, anti-HER2, platelet binding proteins such as annexins, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting moieties. Also, anti-EGF receptor antibodies, which internalize following binding to the receptor and traffic to the nucleus to an extent, are preferred targeting moieties for use in the

present invention to facilitate delivery of Auger emitters and nucleus binding drugs to target cell nuclei. Oligonucleotides, e.g., antisense oligonucleotides that are complementary to portions of target cell nucleic acids (DNA or RNA), are also useful as targeting moieties in the practice of the present invention. Oligonucleotides binding to cell surfaces are also useful. Analogs of the above-listed targeting moieties that retain the capacity to bind to a defined target cell population may also be used within the claimed invention. In addition, synthetic targeting moieties may be designed.

Functional equivalents of the aforementioned molecules are also useful as targeting moieties of the present invention. One targeting moiety functional equivalent is a "mimetic" compound, an organic chemical construct designed to mimic the proper configuration and/or orientation for targeting moiety-target cell binding. Another targeting moiety functional equivalent is a short polypeptide designated as a "minimal" polypeptide, constructed using computer-assisted molecular modeling and mutants having altered binding affinity, which minimal polypeptides exhibit the binding affinity of the targeting moiety.

Preferred targeting moieties of the present invention are antibodies (polyclonal or monoclonal), peptides, oligonucleotides or the like. Polyclonal antibodies useful in the practice of the present invention are polyclonal (Vial and Callahan, *Univ. Mich. Med. Bull.*, 20: 284–6, 1956), affinity-purified polyclonal or fragments thereof (Chao et al., *Res. Comm. in Chem. Path. & Pharm.*, 9: 749–61, 1974).

Monoclonal antibodies useful in the practice of the present invention include whole antibody and fragments thereof. Such monoclonal antibodies and fragments are producible in accordance with conventional techniques, such as hybridoma synthesis, recombinant DNA techniques and protein synthesis. Useful monoclonal antibodies and fragments may be derived from any species (including humans) or may be formed as chimeric proteins which employ sequences from more than one species. See, generally, Kohler and Milstein, *Nature*, 256: 495–97, 1975; *Eur. J. Immunol.*, 6: 511–19, 1976.

Human monoclonal antibodies or "humanized" murine antibody are also useful as targeting moieties in accordance with the present invention. For example, murine-monoclonal antibody may be "humanized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) or the complementarity determining regions thereof with the nucleotide sequence encoding a human constant domain region and an Fc region, e.q, in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Some murine residues may also be retained within the human variable region framework domains to ensure proper target site binding characteristics. Humanized targeting moieties are recognized to decrease the immunoreactivity of the antibody or polypeptide in the host recipient, permitting an increase in the half-life and a reduction in the possibility of adverse immune reactions.

Types of active agents (diagnostic or therapeutic) useful herein include toxins, anti-tumor agents, drugs and radionuclides. Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting protein. However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the toxin A chain-targeting protein conjugate, as compared to the corresponding holotoxin-targeting protein conjugate.

Preferred toxins in this regard include holotoxins, such as abrin, ricin, modeccin, Pseudomonas exotoxin A, Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of Pseudomonas exotoxin A, Diphtheria toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Extremely highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention.

Preferred drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide and cis-platinum, as well as other conventional chemotherapeutics as described in *Cancer: Principles and Practice of Oncology*, 2d ed., V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg, J. B. Lippincott Co., Philadelphia, Pa., 1985, Chapter 14. A particularly preferred drug within the present invention is a trichothecene.

Trichothecenes are drugs produced by soil fungi of the class *Fungi imperfecti* or isolated from *Baccharus megapotamica* (Bamburg, J. R. *Proc. Molec. Subcell. Biol.* 8:41–110, 1983; Jarvis & Mazzola, *Acc. Chem. Res.* 15:338–395, 1982). They appear to be the most toxic molecules that contain only carbon, hydrogen and oxygen (Tamm, C. *Fortschr. Chem. Ora. Naturst.* 31:61–117, 1974). They are all reported to act at the level of the ribosome as inhibitors of protein synthesis at the initiation, elongation, or termination phases.

There are two broad classes of trichothecenes: those that have only a central sesquiterpenoid structure and those that have an additional macrocyclic ring (simple and macrocyclic trichothecenes, respectively). The simple trichothecenes may be subdivided into three groups (i.e., Group A, B, and C) as described in U.S. Pat. Nos. 4;744,981 and 4,906,452 (incorporated herein by reference). Representative examples of Group A simple trichothecenes include: Scirpene, Roridin C, dihydrotrichothecene, Scirpen-4, 8-diol, Verrucarol, Scirpentriol, T-2 tetraol, pentahydroxyscirpene, 4-deacetylneosolaniol, trichodermin, deacetylcalonectrin, calonectrin, diacetylverrucarol, 4-monoacetoxyscirpenol, 4,15-diacetoxyscirpenol, 7-hydroxydiacetoxyscirpenol, 8-hydroxydiacetoxy-scirpenol (Neosolaniol), 7,8-dihydroxydiacetoxyscirpenol, 7-hydroxy-8-acetyldiacetoxyscirpenol, 8-acetylneosolaniol, NT-1, NT-2, HT-2, T-2, and acetyl T-2 toxin.

Representative examples of Group B simple trichothecenes include: Trichothecolone, Trichothecin, deoxynivalenol, 3-acetyldeoxynivalenol, 5-acetyldeoxynivalenol, 3,15-diacetyldeoxynivalenol, Nivalenol, 4-acetylnivalenol (Fusarenon-X), 4,15-idacetylnivalenol, 4,7,15-triacetylnivalenol, and tetra-acetylnivalenol. Representative examples of Group C simple trichothecenes include: Crotocol and Crotocin. Representative macrocyclic trichothecenes include Verrucarin A, Verrucarin B, Verrucarin J (Satratoxin C), Roridin A, Roridin D, Roridin E (Satratoxin D), Roridin H, Satratoxin F, Satratoxin G, Satratoxin H, Vertisporin, Mytoxin A, Mytoxin C, Mytoxin B, Myrotoxin A, Myrotoxin B, Myrotoxin C, Myrotoxin D, Roritoxin A, Roritoxin B, and Roritoxin D. In

addition, the general "trichothecene" sesquiterpenoid ring structure is also present in compounds termed "baccharins" isolated from the higher plant *Baccharis megapotamica*, and these are described in the literature, for instance as disclosed by Jarvis et al. (chemistry of Alleopathy, ACS Symposium Series No. 268: ed. A. C. Thompson, 1984, pp. 149–159).

Experimental drugs, such as mercaptopurine, N-methylformamide, 2-amino-1,3,4-thiadiazole, melphalan, hexamethylmelamine, gallium nitrate, 3% thymidine, dichloromethotrexate, mitoguazone, suramin, bromodeoxyuridine, iododeoxyuridine, semustine, 1-(2-chloroethyl) -3-(2, 6-dioxo-3-piperidyl) -1-nitrosourea, N,N'-hexamethylene-bis-acetamide, azacitidine, dibromodulcitol, Erwinia asparaginase, ifosfamide, 2-mercaptoethane sulfonate, teniposide, taxol, 3-deazauridine, soluble Baker's antifol, homoharringtonine, cyclocytidine, acivicin, ICRF-187, spiromustine, levamisole, chlorozotocin, aziridinyl benzoquinone, spirogermanium, aclarubicin, pentostatin, PALA, carboplatin, amsacrine, caracemide, iproplatin, misonidazole, dihydro-5-azacytidine, 4'-deoxy-doxorubicin, menogaril, triciribine phosphate, fazarabine, tiazofurin, teroxirone, ethiofos, N-(2-hydroxyethyl)-2-nitro-1H-imidazole-1-acetamide, mitoxantrone, acodazole, amonafide, fludarabine phosphate, pibenzimol, didemnin B, merbarone, dihydrolenperone, flavone-8-acetic acid, oxantrazole, ipomeanol, trimetrexate, deoxyspergualin, echinomycin, and dideoxycyidine (see *NCI Investigational Drugs, Pharmaceutical Data* 1987, NIH Publication No. 88–2141, Revised November 1987) are also preferred.

Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Radionucliydes are well-known in the art and include ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}y, ^{88}y, ^{97}Ru, ^{100}Pd, ^{101m}Rh, ^{119}Sb, ^{128}Ba, ^{197}Hg, ^{211}At, ^{212}Bi, ^{153}Sm, ^{169}Eu, ^{212}Pb, ^{109}Pd, ^{111}In, ^{67}Ga, ^{68}Ga, ^{64}Cu, ^{67}Cu, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O, ^{166}HO and ^{18}F. Preferred therapeutic radionuclides include ^{188}Re, ^{186}Re, ^{203}Pb, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au and ^{199}Ag, ^{166}HO or ^{177}Lu.

Other anti-tumor agents, e.q., agents active against proliferating cells, are administrable in accordance with the present invention. Exemplary anti-tumor agents include cytokines, such as IL-2, tumor necrosis factor or the like, lectin inflammatory response promoters (selectins), such as L-selectin, E-selectin, P-selectin or the like, and like molecules.

The galactose cluster reagents may be useful for gene delivery to the liver. Oligonucleotide sequences which might be delivered in accordance with this aspect of the present invention include transcriptionally active gene sequences and gene sequences useful in the antisense format as therapeutic agents. Delivery of genes that are transcriptionally active is particularly advantageous as the liver is very metabolic and receives a large volume of cardiac blood flow output. Genes expressed in the liver, transiently or chronically, and secreted into the circulation will readily perfuse the body. Consequently, delivery of oligonucleotide sequences to the liver may serve to alleviate liver disorders, to address poisoning by hepatotoxic agents in hepatocytes by direct chemical detoxification, or may serve as a platform for the production of therapeutic agents to address other circulation-accessible ailments.

Preferred active agents for use in diagnosis or treatment of liver ailments include the following: anti-parasitic agents, worming agents, anti-cholesterol agents, antibacterials, fungal agents, gene sequences, vitamins, sulfhydryls (e.g., cysteine, glutathione), chelates (e.g., DTPA), nicotinamide co-factors (e.g., NADH, NADPH, NAD and NADP) glucocorticoids, alcohol/aldehyde dehydrogenase, acyclovir, vidarabine, interferon-alpha, corticosteroids and the like. Such active agents may be conjugated to hexose clusters of the present invention in accordance with techniques similar to those described herein for alternative conjugations of such clusters. One skilled in the art is capable of accomplishing such conjugation in accordance with the teachings herein.

One embodiment of the present invention involves the preparation and use of bispecific agents for use in clearance of previously administered molecules or toxic or potentially toxic molecules present in a patient's circulation or extravascular fluid space. Previously administered molecules may include active agent-containing conjugates, targeting moiety-receptor conjugates (e.g., monoclonal antibody or fragment-ligand or anti-ligand conjugates) or the like. In this-circumstance, the hepatic-directed molecule of the present invention is employed to clear the previously administered molecule from non-target sites.

Preferred hepatic-directed molecules of the present invention are present in the circulation and are capable of penetrating the extravascular fluid space. Consequently, previously administered compounds that are present in the circulation or in the extravascular fluid space are accessible to hepatic-directed compounds of the present invention. Circulating compounds are removed via association with hepatic-directed compound and removal via liver receptors. Previously administered compounds, present in extravascular fluid space but not associated with a target cell or epitope, are removed via liver receptors as such compounds are diffused back into the circulation in association with hepatic-directed compounds. Residual hepatic-directed compound which may become bound to a targeted agent (targeting moiety-anti-ligand conjugate, for example) should dissociate over time, thereby providing access to the targeted agent for subsequently administered active agent designed to localize thereto.

Toxic or potentially toxic molecules that may be removed from a recipient's circulation or extravascular fluid space include: chemotherapeutics (e.g., alkylators), heavy metals and the like. Binding moieties capable of associating with toxic or potentially toxic molecules resident in the recipient's circulation or extravascular fluid space include antibodies or fragments thereof directed to epitopes that are characteristic of such toxin or potential toxin. Other useful binding moieties include oligonucleotides, any ligands or anti-ligands in pretargeting embodiments of the present invention.

In pretargeting aspects of the present invention wherein the binding moiety is employed to remove a targeting moiety-ligand or anti-ligand conjugate from the recipient's circulation and/or extravascular fluid space, characteristics of useful binding moieties are discussed below. The binding between the binding moiety of the hepatic-directed compounds of the present invention and the molecule to be cleared from the circulation or extravascular fluid space need only be transient, i.e., for a sufficient amount of time to clear the molecule from circulation or extravascular fluid space to the liver. Under these circumstances, the hepatic-directed molecule of the present invention is employed to remove the toxic or potentially toxic molecule from the patient's circulation or extravascular fluid space.

In general, the binding constant characterizing the interaction of the binding moiety of the hepatic-directed com-

pound and the molecule to be bound thereby should be low enough to keep short the residence time of the hepatic-directed moiety at target sites. Also, the binding constant must be sufficiently high to capture the molecule to be bound and traffic that molecule to the liver. Selection of the ideal binding constant for the binding moieties employed in hepatic-directed compounds of the present invention depends upon factors including:

(i) Rate of clearance of the in vivo-associated construct (e.g., monoclonal antibody-anti-ligand-ligand-galactose cluster) by the liver; and

(ii) Time before an active agent-containing conjugate is administered (in embodiments of the present invention wherein hepatic-directed compounds are employed to clear previously administered moieties).

With respect to criterion (i), the faster the rate of clearance, the lower (weaker) the binding constant needs to be. With respect to criterion, (ii), the greater the amount of time between administration of the hepatic-directed compound and the active agent-containing conjugate, the greater (stronger) the binding constant can be as more time is available for dissociation of the binding moiety from targeted constructs. For clinical convenience, a relatively short time interval and, therefore, a somewhat weaker binding constant are preferred

Binding moieties of the present invention include ligands, anti-ligands, and other target epitope-recognizing moieties. One skilled in the art can substitute acceptable moieties for the binding moieties discussed specifically herein. Preferred binding moieties are characterized by a molecular weight of a Fab fragment of a monoclonal antibody or lower. Such binding moieties may also be modified to include suitable functional groups to allow for attachment of other molecules of interest, e:g., peptides, proteins, nucleotides, and other small molecules.

A recognized disadvantage associated with in vivo administration of targeting moiety-radioisotopic conjugates for imaging or therapy is localization of the attached radioactive agent at both non-target and target sites. Until the administered radiolabeled conjugate clears from the circulation, normal organs and tissues are transitorily exposed to the attached radioactive agent. For instance, radiolabeled whole antibodies that are administered in vivo exhibit relatively slow blood clearance; maximum target site localization generally occurs 1–3 days post-administration. Generally, the longer the clearance time of the conjugate from the circulation, the greater the radioexposure of non-target organs. Therapeutic drugs, administered alone or as targeted conjugates, are accompanied by similar disadvantages.

One method for reducing non-target tissue exposure to a diagnostic or therapeutic agent involves "pretargeting" the targeting moiety at a target site, and then subsequently administering a rapidly clearing diagnostic or therapeutic agent conjugate that is capable of binding to the "pretargeted" targeting moiety at the target site. A description of some embodiments of the pretargeting technique may be found in U.S. Pat. No. 4,863,713 (Goodwin et al.).

"Two-step" pretargeting procedures feature targeting moiety-ligand or targeting moiety-anti-ligand administration, followed by administration of active agent conjugated to the opposite member of the ligand-anti-ligand pair. As an optional step "1.5" in the two-step pretargeting methods of the present invention, a clearing agent (preferably other than ligand or anti-ligand alone) is administered to facilitate the clearance of circulating targeting moiety-containing conjugate.

In the two-step pretargeting approach, the clearing agent preferably does not become bound to the target cell population, either directly or through the previously administered and target cell bound targeting moiety-anti-ligand or targeting moiety-ligand conjugate. An example of two-step pretargeting involves the use of biotinylated human transferrin as a clearing agent for avidin-targeting moiety conjugate, wherein the size of the clearing agent results in liver clearance of transferrin-biotin-circulating avidin-targeting moiety complexes and substantially precludes association with the avidin-targeting moiety conjugates bound at target cell sites. (See, Goodwin, D. A., *Antibod. Immunoconi. Radiopharm.*, 4: 427–34, 1991).

Ligands suitable for use within the present invention include biotin, haptens, lectins, epitopes, dsDNA fragments, enzyme inhibitors and analogs and derivatives thereof. Useful complementary anti-ligands include avidin (for biotin), carbohydrates (for lectins) and antibody, fragments or analogs thereof, including mimetics (for haptens and epitopes) and zinc finger proteins (for dsDNA fragments) and enzymes (for enzyme inhibitors). Preferred ligands and anti-ligands bind to each other with an affinity of at least about k_D 10^9M. Other useful ligand/anti-ligand systems include S-protein/S-peptide, head activator protein (which binds to itself), cystatin-C/cathepsin B, and the like.

One preferred chelate system for use in the practice of the present invention is based upon a 1,4,7,10-tetraazacyclododecane-N, N', N", N'"-tetra acetic acid (DOTA) construct. Because DOTA strongly binds Y-90 and other radionuclides, it has been proposed for use in radioimmunotherapy. For therapy, it is very important that the radionuclide be stably bound within the DOTA chelate and that the DOTA chelate be stably attached to an effector, such as a ligand or an anti-ligand.

The strategy for design of the DOTA-containing molecules and conjugates for use in the practice of embodiments of the present invention wherein the effector is biotin involved three primary considerations:

1) in vivo stability (including biotinidase and general peptidase activity resistance), with an initial acceptance criterion of 100% stability for 1 hour;

2) renal excretion; and

3) ease of synthesis.

The same or similar criteria are applicable to alternative effectors, as can be readily ascertained by one of ordinary skill in the art.

The DOTA-biotin conjugates that are preferably employed in the practice of the present invention reflect the implementation of one or more of the following strategies:

1) substitution of the carbon adjacent to the cleavage susceptible amide nitrogen;

2) alkylation of the cleavage susceptible amide nitrogen;

3) substitution of the amide carbonyl with an alkyl amino group;

4) incorporation of D-amino acids as well as analogs or derivatives thereof; or

5) incorporation of thiourea linkages.

DOTA-biotin conjugates in accordance with the present invention are described in published PCT Patent Application No. PCT/US93/05406. Methods of preparing preferred embodiments of DOTA-biotin conjugates are described in Example III hereof.

The preferred linkers are useful to produce DOTA-biotin or other DOTA-small molecule, conjugates having one or more of the following advantages:

bind avidin or streptavidin with the same or substantially similar affinity as free biotin;

bind metal M^{+3} ions efficiently and with high kinetic stability;

are excreted primarily through the kidneys into urine;

are stable to endogenous enzymatic or chemical degradation (e.g., bodily fluid amidases, peptidases or the like);

penetrate tissue rapidly and bind to pretargeted avidin or streptavidin; and

are excreted rapidly with a whole body residence half-life of less than about 5 hours.

One component to be administered in a preferred two-step pretargeting protocol is a targeting moiety-anti-ligand or a targeting moiety-ligand conjugate. Streptavidin-proteinaceous targeting moiety conjugates are preferably prepared as described in Example II below, with the preparation involving the steps of: preparation of SMCC-derivitized streptavidin; preparation of DTT-reduced proteinaceous targeting moiety; conjugation of the two prepared moieties; and purification of the monosubstituted or disubstituted (with respect to streptavidin) conjugate from crosslinked (antibody-streptavidin-antibody) and aggregate species and unreacted starting materials. The purified fraction is preferably further characterized by one or more of the following techniques: HPLC size exclusion, SDS-PAGE, immunoreactivity, biotin binding capacity and in vivo studies.

One embodiment of the present invention provides clearing agents having physical properties facilitating use for in vivo complexation and blood clearance of anti-ligand/ligand (e.g., avidin/biotin)-targeting moiety (e.g., antibody) conjugates. These clearing agents are useful in improving the target:blood ratio of targeting moiety conjugate.

Other applications of these clearing agents include lesional imaging or therapy involving blood clots and the like, employing antibody or other targeting vehicle-active agent delivery modalities. For example, efficacious anti-clotting agent provides rapid target localization and high target:non-target targeting ratio. Active agents administered in pretargeting protocols of the present invention using efficient clearing agents are targeted in the desirable manner and are, therefore, useful in the imaging/therapy of conditions such as pulmonary embolism and deep vein thrombosis.

Clearing agents useful in the practice of the present invention preferably exhibit one or more of the following characteristics:

rapid, efficient complexation with targeting moiety-ligand (or anti-ligand) conjugate in vivo;

rapid clearance from the blood of targeting moiety conjugate capable of binding a subsequently administered complementary anti-ligand or ligand containing molecule;

high capacity for clearing (or inactivating) large amounts of targeting moiety conjugate; and

low immunogenicity.

Preferred clearing agents include sugar cluster-bearing moieties. The sugars employed in such clusters are preferably hexoses. Such hexose cluster-bearing clearing agents are molecules that have been derivatized to incorporate a cluster of three or more hexoses (six carbon sugar moieties) recognized by Ashwell receptors or other receptors such as the mannose/N-acetylglucosamine receptor which are associated with endothelial cells and/or Kupffer cells of the liver or the mannose 6-phosphate receptor. Exemplary of such hexoses are galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, pentamannosylphosphate, and the like. Other moieties recognized by Ashwell receptors, including glucose, N-galactosamine, N-acetylgalactosamine, pentamannosyl phosphate, thioglycosides of galactose and, generally, D-galactosides and glucosides or the like may also be used in the practice of the present invention. Galactose is the prototypical clearing agent hexose derivative for the purposes of this description.

Exposed galactose residues of the galactose cluster direct the clearing agent to rapid clearance by endocytosis into the liver through specific receptors therefor (Ashwell receptors). These receptors bind the clearing agent, and induce endocytosis into the hepatocyte, leading to fusion with a lysosome and recycle of the receptor back to the cell surface. This clearance mechanism is characterized by high efficiency, high capacity and rapid kinetics.

Clearing agents previously developed incorporated human serum albumin (HSA) as follows:

$(Hexose)_m$—Human Serum Albumin (HSA)—$(Ligand)_n$,

wherein n is an integer from 1 to about 10 and m is an integer from 1 to about 45 and wherein the hexose is recognized by Ashwell receptors.

The galactose cluster-bearing clearing agents of the present invention are preferably capable of (1) rapidly and efficiently complexing with the relevant ligand- or anti-ligand-containing conjugates via ligand-anti-ligand affinity; and (2) clearing such complexes from the blood via the galactose receptor, a liver specific degradation system, as opposed to aggregating into complexes that are taken up by the generalized RES system, including the lung and spleen. Additionally, the rapid kinetics of galactose-mediated liver uptake, coupled with the affinity of the ligand-anti-ligand interaction, allow the use of intermediate or even low molecular weight carriers.

Clearing agent evaluation experimentation involving galactose- and biotin-derivatized clearing agents is detailed in Example IV. The specific clearing agent examined during the Example IV experimentation are human serum albumin derivatized with galactose and biotin and a 70,000 dalton molecular weight dextran derivatized with both biotin and galactose. The experimentation showed that proteins and polymers are derivatizable to contain both galactose and biotin and that the resultant derivatized molecule is effective in removing circulating streptavidin-protein conjugate from the serum of the recipient. Biotin loading was varied to determine the effects on both clearing the blood pool of circulating avidin-containing conjugate and the ability to deliver a subsequently administered biotinylated isotope to a target site recognized by the streptavidin-containing conjugate. The effect of relative doses of the administered components with respect to clearing agent efficacy was also examined. Experimentation comparing such clearing agents to those hexose cluster-bearing moieties of the present invention is set forth in Example VI below.

The small molecule clearing agents are superior to the proteinaceous clearing agents from cost, regulatory and characterization perspectives. More specifically, the small molecule clearing agents are preparable from available or easily synthesizable components and are amenable to more precise characterization. In addition, if biotin release from the proteinaceous clearing agent is determined to be problematic, such release can be avoided or the impact of such release minimized using a small molecule clearing agent incorporating a highly stable biotin linker or incorporating a lower affinity biotin analog, respectively.

The present invention provides sugar cluster-bearing clearing agents that incorporate ligand derivatives or anti-ligand derivatives, wherein such derivatives exhibit a lower affinity for the complementary ligand/anti-ligand pair member than the native form of the compound (i.e., lower affinity

ligands or anti-ligands). In embodiments of the present invention employing a biotin-avidin or biotin-streptavidin ligand/anti-ligand pair, preferred sugar cluster-bearing clearing agents incorporate either lower affinity biotin (which exhibits a lower affinity for avidin or streptavidin than native biotin) or lower affinity avidin or a streptavidin (which exhibits a lower affinity for biotin than native avidin or streptavidin).

Sugar cluster-bearing clearing agents that employ a ligand or anti-ligand moiety that is complementary to the ligand/anti-ligand pair member (previously administered in conjunction with the targeting moiety) are useful in the practice of the present invention. When such clearing agents localize to hepatocytes, they are generally rapidly degraded. This degradation liberates a quantity of free ligand or free anti-ligand into the circulation. This bolus release of ligand or anti-ligand may compete for binding sites of targeting moiety-ligand or targeting moiety-anti-ligand with subsequently administered active agent-ligand or active agent-anti-ligand conjugate.

This competition can be addressed by using a sugar cluster-bearing clearing agent incorporating a lower affinity ligand or anti-ligand. In other words, the ligand or anti-ligand employed in the structure of the clearing agent more weakly binds to the complementary ligand/anti-ligand pair member than native ligand or anti-ligand. Consequently, lower affinity ligand or anti-ligand derivatives that bind to target-localized targeting moiety-anti-ligand or targeting moiety-ligand conjugate may be displaced by the subsequently administered, active agent-native (or higher binding affinity ligand) or active agent-native (or higher binding affinity) anti-ligand conjugate.

In two-step pretargeting protocols employing the biotin-avidin or biotin-streptavidin ligand-anti-ligand pair, lower affinity biotin, lower affinity avidin or lower affinity streptavidin may be employed. Exemplary lower affinity biotin molecules, for example, exhibit the following properties: bind to avidin or streptavidin with an affinity less than that of native biotin (10^{-15}); retain specificity for binding to avidin or streptavidin; are non-toxic to mammalian recipients; and the like. Exemplary lower affinity avidin or streptavidin molecules, for example, exhibit the following properties: bind to biotin with an affinity less than native avidin or streptavidin; retain specificity for binding to biotin; are non-toxic to mammalian recipients; and the like.

Exemplary lower affinity biotin molecules include 2'-thiobiotin; 2'-iminobiotin; 1'-N-methoxycarbonyl-biotin; 3'-N-methoxycarbonylbiotin; 1-oxy-biotin; 1-oxy-2'-thiobiotin; 1-oxy-2'-iminobiotin; 1-sulfoxide-biotin; 1-sulfoxide-2'-thiobiotin; 1-sulfoxide-2'iminobiotin; 1-sulfone-biotin; 1-sulfone-2'-thio-biotin; 1-sulfone-2'-iminobiotin; imidazolidone derivatives such as desthiobiotin (d and dl optical isomers), dl-desthiobiotin methyl ester, dl-desthiobiotinol, D-4-n-hexyl-imidazolidone, L-4-n-hexylimidazolidone, dl-4-n-butyl-imidazolidone, dl-4-n-propylimidazolidone, dl-4-ethyl-imidazolidone, dl-4-methylimidazolidone, imidazolidone, dl-4,5-dimethylimidazolidone, meso-4,5-dimethylimidazolidone, dl-norleucine hydantoin, D-4-n-hexyl-2-thionoimidazolidine, d-4-n-hexyl-2-imino-imidazolidine and the like; oxazolidone derivatives such as D-4-n-hexyloxazolidone, D-5-n-hexyloxazolidone and the like; [5-(3,4-diamino-thiophan-2-yl] pentanoic acid; lipoic acid; 4-hydroxy-azobenzene-2'-carboxylic acid; and the like. Preferred lower affinity biotin molecules for use in the practice of the present invention are 2'-thiobiotin, desthiobiotin, 1-oxy-biotin, 1-oxy-2'-thiobiotin, 1-sulfoxide-biotin, 1-sulfoxide-2'-thiobiotin, 1-sulfone-biotin, 1-sulfone-2'-thiobiotin, lipoic acid and the like. These exemplary lower affinity biotin molecules may be produced substantially in accordance with known procedures therefor. Conjugation of the exemplary lower affinity biotin molecules to sugar cluster directors proceeds substantially in accordance with procedures described herein in regard to biotin conjugation.

Much has been reported about the binding affinity of different biotin analogs to avidin. Based upon what is known in the art, the ordinary skilled artisan could readily select or use known techniques to ascertain the respective binding affinity of a particular biotin analog to streptavidin, avidin or a derivative thereof.

The present invention further provides methods of increasing active agent localization at a target cell site of a mammalian recipient, which methods include:

- administering to the recipient a first conjugate comprising a targeting moiety and a member of a ligand-anti-ligand binding pair;
- thereafter administering to the recipient a clearing agent bearing a sugar cluster capable of directing the clearance of circulating first conjugate via hepatocyte receptors of the recipient, wherein the clearing agent incorporates lower affinity complementary member of the ligand-anti-ligand binding pair; and
- subsequently administering to the recipient a second conjugate comprising an active agent and a ligand/anti-ligand binding pair member, wherein the second conjugate binding pair member is complementary to that of the first conjugate and, preferably, constitutes a native or high affinity form of the member.

Clearing agents of the present invention may be administered in single or multiple doses or via continuous infusion. A single dose of biotinylated clearing agent, for example, produces a rapid decrease in the level of circulating targeting moiety-streptavidin, followed by a small increase in that level, presumably caused, at least in part, by re-equilibration of targeting moiety-streptavidin within the recipient's physiological compartments. A second or additional clearing agent doses may then be employed to provide supplemental clearance of targeting moiety-streptavidin. Alternatively, clearing agent may be infused intravenously for a time period sufficient to clear targeting moiety-streptavidin in a continuous manner.

Other types of clearing agents and clearance systems are also useful in the practice of the present invention to remove circulating targeting moiety-ligand or -anti-ligand conjugate from the recipient's circulation. Particulate-based clearing agents, for example, are discussed in Example I. Such particulate-based clearing agents can be employed in conjunction with sugar clusters to provide hepatic-directed compounds of the present invention.

One embodiment of the present invention in which rapid acting sugar cluster director-bearing clearing agents are useful is in the delivery of Auger emitters, such as I-125, I-123, Er-165, Sb-119, Hg-197, Ru-97, Tl-201 and I-125 and Br-77, or nucleus-binding drugs to target cell nuclei. In these embodiments of the present invention, targeting moieties that localize to internalizing receptors on target cell surfaces are employed to deliver a targeting moiety-containing conjugate (i.e., a targeting moiety-anti-ligand conjugate in the preferred two-step protocol) to the target cell population. Such internalizing receptors include EGF receptors, transferrin receptors, HER2 receptors, IL-2 receptors, other interleukins and cluster differentiation receptors, somatostatin receptors, other peptide binding receptors and the like.

After the passage of a time period sufficient to achieve localization of the conjugate to target cells, but insufficient

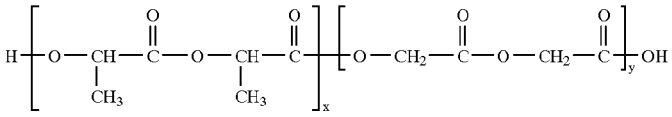
to induce internalization of such targeted conjugates by those cells through a receptor-mediated event, a rapidly acting sugar cluster director-bearing clearing agent is administered. In a preferred two-step protocol, an active agent-containing ligand or anti-ligand conjugate, such as a biotin-Auger. emitter or a biotin-nucleus acting drug, is administered as soon as the clearing agent has been given an opportunity to complex with circulating targeting moiety-containing conjugate, with the time lag between clearing agent and active agent administration being less than about 24 hours. In this manner, active agent is readily internalized through target cell receptor-mediated internalization. While circulating Auger emitters are thought to be non-toxic, the rapid, specific targeting afforded by the pretargeting protocols of the present invention increases the potential of shorter half-life Auger emitters, such as I-123, which is available and capable of stable binding.

The invention is further described through presentation of the following examples. These examples are offered by way of illustration, and not by way of limitation.

dosage form through covalent or non-covalent modalities as set forth herein to provide accessible ligand or anti-ligand for binding to its previously administered circulating binding pair member.

Preferable particulate clearing agents of the present invention are biodegradable or non-biodegradable microparticulates. More preferably, the particulate clearing agents are formed of a polymer containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning.

Polymers derived from the condensation of alpha hydroxycarboxylic acids and related lactones are more preferred for use in the present invention. A particularly preferred moiety is formed of a mixture of thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components, such as poly (lactide-co-glycolide). An exemplary structure, a random poly(DL-lactide-co-glycolide), is shown.below, with the values of x and y being manipulable by a practitioner in the art to achieve desirable microparticulate properties.



EXAMPLE I

Particulate Clearing Agents

Clearance of chimeric monoclonal antibody-avidin is facilitated by administration of a particulate-type clearing agent (e.g., a polymeric particle having a plurality of biotin molecules bound thereto). Such a particulate clearing agent preferably constitutes a biodegradable polymeric carrier having a plurality of biotin molecules bound thereto. Particulate clearing agents of the present invention exhibit the capability of binding to circulating administered conjugate and removing that conjugate from the recipient. Particulate clearing agents of this aspect of the present invention may be of any configuration suitable for this purpose. Preferred particulate clearing agents exhibit one or more of the following characteristics:

- microparticulate (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, with from about 0.5 to about 2 micrometers more preferred), free flowing powder structure;
- biodegradable structure designed to biodegrade over a period of time between from about 3 to about 180 days, with from about 10 to about 21 days more preferred, or non-biodegradable structure;
- biocompatible with the recipients physiology over the course of distribution, metabolism and excretion of the clearing agent, more preferably including biocompatible biodegradation products;
- and capability to bind with one or more circulating conjugates to facilitate the elimination or removal thereof from the recipient through one or more binding moieties (preferably, the complementary member of the ligand/anti-ligand pair). The total molar binding capacity of the particulate clearing agents depends upon the particle size selected and the ligand or anti-ligand substitution ratio. The binding moieties are capable of coupling to the surface structure of the particulate

Other agents suitable for forming particulate clearing agents of the present invention include polyorthoesters and polyacetals (*Polymer Letters*, 18:293, 1980) and polyorthocarbonates (U.S. Pat. No. 4,093,709) and the like.

Preferred lactic acid/glycolic acid polymer containing matrix particulates of the present invention are prepared by emulsion-based processes, that constitute-modified solvent extraction processes such as those described by Cowsar et al., "Poly(Lactide-Co-Glycolide) Microcapsules for Controlled Release of Steroids," *Methods Enzymolocy*, 112:101–116, 1985 (steroid entrapment in microparticulates); Eldridge et al., "Biodegradable and Biocompatible Poly(DL-Lactide-Co-Glycolide) Microspheres as an Adjuvant for Staphylococcal Enterotoxin B Toxoid Which Enhances the Level of Toxin-Neutralizing Antibodies," *Infection and Immunity*, 59:2978–2986, 1991 (toxoid entrapment); Cohen et al., "Controlled Delivery Systems for Proteins Based on Poly(Lactic/Glycolic Acid) Microspheres," *Pharmaceutical Research*, 8(6):713–720, 1991 (enzyme entrapment); and Sanders et al., "Controlled Release of a Luteinizing Hormone-Releasing Hormone Analogue from Poly(D,L-Lactide-Co-Glycolide) Microspheres," *J. Pharmaceutical Science*, 73(9):1294–1297, 1984 (peptide entrapment).

In general, the procedure for forming particulate clearing agents of the present invention involves dissolving the polymer in a halogenated hydrocarbon solvent and adding an additional agent that acts as a solvent for the halogenated hydrocarbon solvent but not for the polymer. The polymer precipitates out from the polymer-halogenated hydrocarbon solution. Following particulate formation, they are washed and hardened with an organic solvent. Water washing and aqueous non-ionic surfactant washing steps follow, prior to drying at room temperature under vacuum.

For biocompatibility purposes, particulate clearing agents are sterilized prior to packaging, storage or administration. Sterilization may be conducted in any convenient manner therefor. For example, the particulates can be irradiated with gamma radiation, provided that exposure to such radiation

does not adversely impact the structure or function of the binding moiety attached thereto. If the binding moiety is so adversely impacted, the particulate clearing agents can be produced under sterile conditions.

The preferred lactide/glycolide structure is biocompatible with the mammalian physiological environment. Also, these preferred sustained release dosage forms have the advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

Functional groups required for binding moiety-particulate bonding, are optionally included in the particulate structure, along with the non-degradable or biodegradable polymeric units. Functional groups that are exploitable for this purpose include those that are reactive with ligands or anti-ligands and hexose cluster director reagents, such as carboxyl groups, amine groups, sulfhydryl groups and the like. Preferred binding enhancement moieties include the terminal carboxyl groups of the preferred (lactide-glycolide) polymer containing matrix or the like. A practitioner in the art is capable of selecting appropriate functional groups and monitoring conjugation reactions involving those functional groups.

Advantages garnered through the use of particulate clearing agents of the type described above are as follows:

- particles in the "micron" size range localize in the RES and liver, with galactose derivatization or charge modification enhancement methods for this capability available, and, preferably, are designed to remain in circulation for a time sufficient to perform the clearance function;
- the size of the particulates facilitates central vascular compartment retention thereof, substantially precluding equilibration into the peripheral or extravascular compartment;
- desired substituents for ligand or anti-ligand binding to the particulates can be introduced into the polymeric structure;
- ligand- or anti-ligand-particulate linkages having desired properties (e.g., serum biotinidase resistance thereby reducing the release of biotin metabolite from a particle-biotin clearing agent) and
- multiple ligands or anti-ligands can be bound to the particles to achieve optimal cross-linking of circulating targeting agent-ligand or -anti-ligand conjugate and efficient clearance of cross-linked species. This advantage is best achieved when care is taken to prevent particulate aggregation both in storage and upon in vivo administration.

EXAMPLE II

Targeting Moiety-Anti-Ligand Conjugate for Two-Step Pretargeting In Vivo

A. Preparation of SMCC-Derivitized Streltavidin.

31 mg (0.48 mol) streptavidin was dissolved in 9.0 ml PBS to prepare a final solution at 3.5 mg/ml. The pH of the solution was adjusted to 8.5 by addition of 0.9 ml of 0.5 M borate buffer, pH 8.5. A DMSO solution of SMCC (3.5 mg/ml) was prepared, and 477 l (4.8 mol) of this solution was added dropwise to the vortexing protein solution. After 30 minutes of stirring, the solution was purified by G-25 (PD-10, Pharmacia, Picastaway, N.J.) column chromatography to remove unreacted or hydrolyzed SMCC. The purified SMCC-derivitized streptavidin was isolated (28 mg, 1.67 mg/ml).

B. Preparation of DTT-Reduced NR-LU-10.

To 77 mg NR-LU-10 (0.42 mol) in 15.0 ml PBS was added 1.5 ml of 0.5 M borate buffer, pH 8.5. A DTT solution, at 400 mg/ml (165 1) was added to the protein solution. After stirring at room temperature for 30 minutes, the reduced antibody was purified by G-25 size exclusion chromatography. Purified DTT-reduced NR-LU-10 was obtained (74 mg, 2.17 mg/ml).

C. Coniugation of SMCC-Streptavidin to DTT-Reduced NR-LU-10.

DTT-reduced NR-LU-10 (63 mg, 29 ml, 0.42 mol) was diluted with 44.5 ml PBS. The solution of SMCC-streptavidin (28 mg, 17 ml, 0.42 mol) was added rapidly to the stirring solution of NR-LU-10. Total protein concentration in the reaction mixture was 1.0 mg/ml. The progress of the reaction was monitored by HPLC (Zorbax® GF-250, available from MacMod). After approximately 45 minutes, the reaction was quenched by adding solid sodium tetrathionate to a final concentration of 5 mM.

D. Purification of Conjugate.

For small scale reactions, monosubstituted or disubstituted (with regard to streptavidin) conjugate was obtained using HPLC Zorbax (preparative) size exclusion chromatography. The desired monosubstituted or disubstituted conjugate product eluted at 14.0–14.5 min (3.0 ml/min flow rate), while unreacted NR-LU-10 eluted at 14.5–15 min and unreacted derivitized streptavidin eluted at 19–20 min.

For larger scale conjugation reactions, monosubstituted or disubstituted adduct is isolatable using DEAE ion exchange chromatography. After concentration of the crude conjugate mixture, free streptavidin was removed therefrom by eluting the column with 2.5% xylitol in sodium borate buffer, pH 8.6. The bound unreacted antibody and desired conjugate were then sequentially eluted from the column using an increasing salt gradient in 20 mM diethanolamine adjusted to pH 8.6 with sodium hydroxide.

E. Characterization of Coniugate.

1. HPLC size exclusion was conducted as described above with respect to small scale purification.

2. SDS-PAGE analysis was performed using 5% polyacrylamide gels under non-denaturing conditions. Conjugates to be evaluated were not boiled in sample buffer containing SDS to avoid dissociation of streptavidin into its 15 kD subunits. Two product bands were observed on the gel, which correspond to the mono- and di- substituted conjugates.

3. Immunoreactivity was assessed, for example, by competitive binding ELISA as compared to free antibody. Values obtained were within 10% of those for the free antibody.

4. Biotin binding capacity was assessed, for example, by titrating a known quantity of conjugate with p-[I-125] iodobenzoylbiocytin. Saturation of the biotin binding sites was observed upon addition of 4 equivalences of the labeled biocytin.

5. In vivo studies are useful to characterize the reaction product, which studies include, for example, serum clearance profiles, ability of the conjugate to target antigen-positive tumors, tumor retention of the conjugate over time and the ability of a biotinylated molecule to bind streptavidin conjugate at the tumor.

These data facilitate determination that the synthesis resulted in the formation of a 1:1 streptavidin-NR-LU-10 whole antibody conjugate that exhibits blood clearance properties similar to native NR-LU-10 whole antibody, and tumor uptake and retention properties at least equal to native NR-LU-10.

For example, FIG. 1 depicts the tumor.uptake profile of the NR-LU-10-streptavidin conjugate (LU-10-StrAv) in comparison to a control profile of native NR-LU-10 whole antibody. LU-10-StrAv was radiolabeled on the streptavidin

component only, giving a clear indication that LU-10-StrAv localizes to target cells as efficiently as NR-LU-10 whole antibody itself.

EXAMPLE III

Synthesis of DOTA-Biotin Conjugates

A. Synthesis of Nitro-Benzyl-DOTA.

The synthesis of aminobenzyl-DOTA was conducted substantially in accordance with the procedure of McMurry et al., *Bioconlurate Chem.*, 3: 108–117, 1992. The critical step in the prior art synthesis is the intermolecular cyclization between disuccinimidyl N-(tert-butoxycarbonyl) iminodiacetate and N-(2-aminoethyl)-4-nitrophenyl alaninamide to prepare 1-(tert-butoxycarbonyl)-5-(4-nitrobenzyl)-3,6,11-trioxo-1,4,7,10-tetraazacyclododecane. In other words, the critical step is the intermolecular cyclization between the bis-NHS ester and the diamine to give the cyclized dodecane. McMurry et al. conducted the cyclization step on a 140 mmol scale, dissolving each of the reagents in 100 ml DMF and adding via a syringe pump over 48 hours to a reaction pot containing 4 liters dioxane.

A 5× scale-up of the McMurry et al. procedure was not practical in. terms of reaction volume, addition rate and reaction time. Process chemistry studies revealed that the reaction addition rate could be substantially increased and that the solvent volume could be greatly reduced, while still obtaining a similar yield of the desired cyclization product. Consequently on a 30 mmol scale, each of the reagents was dissolved in 500 ml DMF and added via addition funnel over 27 hours to a reaction pot containing 3 liters dioxane. The addition rate of the method employed involved a 5.18 mmol/hour addition rate and a 0.047 M reaction concentration.

B. Synthesis of an N-methyl-alycine Linked Conlucate.

The N-methyl glycine-linked DOTA-biotin conjugate was prepared by an analogous method to that used to prepare D-alanine-linked DOTA-biotin conjugates. N-methyl-glycine (trivial name sarcosine, available from Sigma Chemical Co.) was condensed with biotin-NHS ester in DMF and triethylamine to obtain N-methyl glycyl-biotin. N-methyl-glycyl biotin was then activated with EDCI and NHS. The resultant NHS ester was not isolated and was condensed in situ with DOTA-aniline and excess pyridine. The reaction solution was heated at 60° C. for 10 minutes and then evaporated. The residue was purified by preparative HPLC to give [(N-methyl-N-biotinyl)-N-glycyl]-aminobenzyl-DOTA.

1. Preparation of (N-methyl)glycyl biotin. DMF (8.0 ml) and triethylamine (0.61 ml, 4.35 mmol) were added to solids N-methyl glycine (182 mg, 2.05 mmol) and N-hydroxy-succinimidyl biotin (500 mg, 1.46 mmol). The mixture was heated for 1 hour in an oil bath at 85° C. during which time the solids dissolved producing a clear and colorless solution. The solvents were then evaporated. The yellow oil residue was acidified with glacial acetic acid, evaporated and chromatographed on a 27 mm column packed with 50 g silica, eluting with 30% MeOH/EtOAc 1% HOAc to give the product as a white solid (383 mg) in 66% yield.

II-NMR (DMSO): 1.18–1.25 (m, 6II, $(CII_2)_3$), 2.15, 2.35 (2 t's, 2H, CH_2CO), 2.75 (m, 2H, SCH_2), 2.80, 3.00 (2 s's, 3H, NCH_3), 3.05–3.15 (m, 1H, SCH), 3.95, 4.05 (2 s's, 2H, CH_2N), 4.15, 4.32 (2 m's, 2H, 2CHN's), 6.35 (s, NH), 6.45 (s, NH).

2. Preparation of [(N-methyl-N-biotinyl)glycyl] aminobenzyl-DOTA. N-hydroxysuccinimide (10 mg, 0.08 mmol) and EDCI (15 mg, 6.08 mmol) were added to a solution of (N-methylglycyl) biotin (24 mg, 0.08 mmol) in DMF (1.0 ml). The solution was stirred at 23° C. for 64 hours. Pyridine (0.8 ml) and aminobenzyl-DOTA (20 mg, 0.04 mmol) were added. The mixture was heated in an oil bath at 63° C. for 10 minutes, then stirred at 23° C. for 4 hours. The solution was evaporated. The residue was purified by preparative HPLC to give the product as an off white solid (8 mg, 0.01 mmol) in 27% yield.

H-NMR (D_2O): 1.30–1.80 (m, 6H), 2.40, 2.55 (2 t's, 2H, CH_2CO), 2.70–4.2 (complex multiplet), 4.35 (m, CHN), 4.55 (m, CHN), 7.30 (m, 2H, benzene hydrogens), 7.40 (m, 2H, benzene hydrogens).

EXAMPLE IV

Clearing Agent Evaluation Experimentation

A. Galactose- and Biotin-Derivatization of Human Serum Albumin (HSA).

HSA was evaluated because it exhibits the advantages of being both inexpensive and non-immunogenic. IISA was derivatized with varying levels of biotin (1-about 9 biotins/molecule) via analogous chemistry to that previously described with respect to AO. More specifically, to a solution of HSA available from Sigma Chemical Co. (5–10 mg/ml in PBS) was added 10% v/v 0.5M sodium borate buffer, pH 8.5, followed by dropwise addition of a DMSO solution of NHS-LC-biotin (Sigma Chemical Co.) to the stirred solution at the desired molar offering (relative molar equivalents of reactants). The final percent DMSO in the reaction mixture should not exceed 5%. After stirring for 1 hour at room temperature, the reaction was complete. A 90% incorporation efficiency for biotin on HSA was generally observed. As a result, if 3 molar equivalences of the NHS ester of LC-biotin was introduced, about 2.7 biotins per HSA molecule were obtained. Unreacted biotin reagent was removed from the biotin-derivatized HSA using G-25 size exclusion chromatography. Alternatively, the crude material may be directly galactosylated. The same chemistry is applicable for biotinylating- non-previously biotinylated dextran.

HSA-biotin was then derivatized with from 12 to 45 galactoses/molecule. Galactose derivatization of the biotinylated HSA was performed according to the procedure of Lee, et al., *Biochemistry*, 15: 3956, 1976. More specifically, a 0.1M methanolic solution of cyanomethyl-2,3,4,6-tetra-O-acetyl-1-thio-D-galactopyranoside was prepared and reacted with a 10% v/v 0.1M NaOMe in methanol for 12 hours to generate the reactive galactosyl thioimidate. The galactosylation of biotinylated HSA began by initial evaporation of the anhydrous methanol from a 300 fold molar excess of reactive thioimidate. Biotinylated HSA in PBS, buffered with 10% v/v 0.5M sodium borate, was added to the oily residue. After stirring at room temperature for 2 hours, the mixture was stored at 4° C. for 12 hours. The galactosylated HSA-biotin was then purified by G-25 size exclusion chromatography or by buffer,exchange to yield the desired product. The same chemistry is exploitable to galactosylating dextran. The incorporation efficiency of galactose on HSA is approximately 10%.

70 micrograms of Galactose-HSA-Biotin (G-HSA-B), with 12–45 galactose residues and 9 biotins, was administered to mice which had been administered 200 micrograms of StrAv-MAb or 200 microliters of PBS 24 hours earlier. Results indicated that G-HSA-B is effective in removing StrAv-MAb from circulation. Also, the pharmacokinetics of G-HSA-B is unperturbed and rapid in the presence or absence of circulating MAb-StrAv.

B. Non-Protein Clearing Agent.

A commercially available form of dextran, molecular weight of 70,000 daltons, pre-derivatized with approxi-

mately 18 biotins/molecule and having an equivalent number of free primary amines was studied. The primary aminie moieties were derivatized with a galactosylating reagent, substantially in accordance with the procedure therefor described above in the discussion of HSA-based clearing agents, at a level of about 9 galactoses/molecule. The molar equivalence offering ratio of galactose to HSA was about 300:1, with about one-third of the galactose being converted to active form. 40 Micrograms of galactose-dextran-biotin (GAL-DEX-BT) was then injected i.v. into one group of mice which had received 200 micrograms MAb-StrAv conjugate intravenously 24 hours earlier, while 80 micrograms of GAL-DEX-BT was injected into other such mice. GAL-DEX-BT was rapid and efficient at clearing StrAv-MAb conjugate, removing over 66% of circulating conjugate in less than 4 hours after clearing agent administration. An equivalent effect was seen at both clearing agent doses, which correspond to 1.6 (40 micrograms) and 3.2 (80 micrograms) times the stoichiometric amount of circulating StrAv conjugate present.

C. Dose Ranginc for G-HSA-B Clearing Agent.

Dose ranging studies followed the following basic format:

200 micrograms MAb-StrAv conjugate administered;

24 hours later, clearing agent administered; and

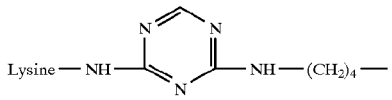
2 hours later, 5.7 micrograms PIP-biocytin administered.

Dose ranging studies were performed with the G-HSA-B clearing agent, starting with a loading of 9 biotins per molecule and 12–45 galactose residues per molecule. Doses of 20, 40, 70 and 120 micrograms were administered 24 hours after a 200 microgram dose of MAb-StrAv conjugate. The clearing agent administrations were followed 2 hours later by administration of 5.7 micrograms of I-131-PIP-biocytin. Tumor uptake and blood retention of PIP-biocytin was examined 44 hours after administration thereof (46 hours after clearing agent administration). The results showed that a nadir in blood retention of PIP-biocytin was achieved by all doses greater than or equal to 40 micrograms of G-HSA-B. A clear, dose-dependent decrease in tumor binding of PIP-biocytin at each increasing dose of G-HSA-B was present, however. Since no dose-dependent effect on the localization of MAb-StrAv conjugate at the tumor was observed, this data was interpreted as being indicative of relatively higher blocking of tumorassociated MAb-StrAv conjugate by the release of biotin from catabolized clearing agent. Similar results to those described earlier for the asialoorosomucoid clearing agent regarding plots of tumor/blood ratio were found with respect to G-HSA-B, in that an optimal balance between blood clearance and tumor retention occurred around the 40 microgram dose.

Because of the relatively large molar amounts of biotin that could be released by this clearing agent at higher doses, studies were undertaken to evaluate the effect of lower levels of biotinylation on the effectiveness of the clearing agent. G-HSA-B, derivatized with either 9, 5 or 2 biotins/molecule, was able to clear MAb-StrAv conjugate from blood at equal protein doses of clearing agent. All levels of biotinylation yielded effective, rapid clearance of MAb-StrAv from blood.

Comparison of these 9-, 5-, and 2-biotin-derivatized clearing agents with a single biotin G-IISA-B clearing agent was carried out in tumored mice, employing a 60 microgram dose of each clearing agent. This experiment showed each clearing agent to be substantially equally effective in blood clearance and tumor retention of MAb-StrAv conjugate 2 hours after clearing agent administration. The G-HSA-B with a single biotin was examined for the ability to reduce binding of a subsequently administered biotinylated small molecule (PIP-biocytin) in blood, while preserving tumor binding of PIP-biocytin to prelocalized MAb-StrAv conjugate. Measured at 44 hours following PIP-biocytin administration, tumor localization of both the MAb-StrAv conjugate and PIP-biocytin was well preserved over a broad dose range of G-HSA-B with one biotin/molecule (90 to 180 micrograms). A progressive decrease in blood retention of PIP-biocytin was achieved by increasing doses of the single biotin G-HSA-B clearing agent, while tumor localization remained essentially constant, indicating that this clearing agent, with a lower level of biotinylation, is preferred. This preference arises because the single biotin G-HSA-B clearing agent is both effective at clearing MAb-StrAv over a broader range of doses (potentially eliminating the need for patient-to-patient titration of optimal dose) and appears to release less competing biotin into the systemic circulation than the same agent having a higher biotin loading level.

Another way in which to decrease the effect of clearing agent-released biotin on active agent-biotin conjugate binding to prelocalized targeting moiety-streptavidin conjugate is to attach the protein or polymer or other primary clearing agent component to biotin using a retention linker. A retention linker has a chemical structure that is resistant to agents that cleave peptide bonds and, optionally, becomes protonated when localized to a catabolizing space, such as a lysosome. Preferred retention linkers of the present invention are short strings of D-amino acids or small molecules having both of the characteristics set forth above. An exemplary retention linker of the present invention is cyanuric chloride, which may be interposed between an epsilon amino group of a lysine of a proteinaceous primary clearing agent component and an amine moiety of a reduced and chemically altered biotin carboxy moiety (which has been discussed above) to form a compound of the structure set forth below.



When the compound shown above is catabolized in a catabolizing space, the heterocyclic ring becomes protonated. The ring protonation prevents the catabolite from exiting the lysosome. In this manner, biotin catabolites containing the heterocyclic ring are restricted to the site(s) of catabolism and, therefore, do not compete with active-agent-biotin conjugate for prelocalized targeting moiety-streptavidin target sites.

Comparisons of tumor/blood localization of radiolabeled PIP-biocytin observed in the G-HSA-B dose ranging studies showed that optimal tumor to background targeting was achieved over a broad dose range (90 to 180 micrograms), with the results providing the expectation that even larger clearing agent doses would also be effective. Another key result of the dose ranging experimentation is that G-HSA-B with an average of only 1 biotin per molecule is presumably only clearing the MAb-StrAv conjugate via the Ashwell receptor mechanism only, because too few biotins are present to cause cross-linking and aggregation of MAb-StrAv conjugates and clearing agents with such aggregates being cleared by the reticuloendothelial system.

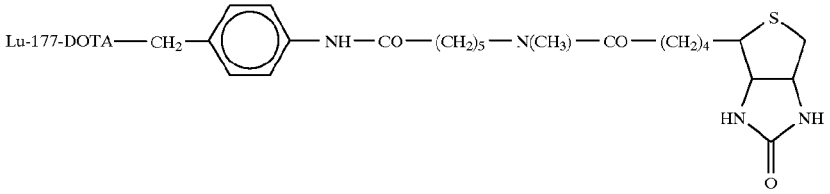
D. Tumor Targeting Evaluation Using G-HSA-B.

The protocol for this experiment was as follows:

Time 0: administer 400 micrograms MAb-StrAv conjugate;

Time 24 hours: administer 240 micrograms of G-HSA-B with one biotin and 12–45 galactoses and

Time 26 hours: administer 6 micrograms of



Lu-177 is complexed with the DOTA chelate using known techniques therefor.

Efficient delivery of the Lu-177-DOTA-biotin small molecule was observed, 20–25% injected dose/gram of tumor. These values are equivalent with the efficiency of the delivery of the MAb-StrAv conjugate. The AUC tumor/AUC blood obtained for this non-optimized clearing agent dose was 300% greater than that achievable by comparable direct MAb-radiolabel administration. Subsequent experimentation has resulted in AUC tumor/AUC blood over 1000% greater than that achievable by comparable conventional MAb-radiolabel administration. In addition, the HSA-based clearing agent is expected to exhibit a low degree of immunogenicity in humans.

EXAMPLE V

Small Molecule Clearing Agent Preparation

This procedure is shown schematically in FIG. **2**.

Methyl 6-bromohexanoate. To a 1 L round bottom flask, charged with 20 g (102.5 mmol) of 6-bromohexanoic acid and 500 mL of methanol, was bubbled hydrogen chloride gas for 2–3 minutes. The mixture was stirred at room temperature for 4 hours and concentrated to afford 21.0 g of the product as a yellow oil (99%): ^{1}II-NMR (200MIIz, d_6-DMSO); 3.57 (s, 3H), 3.51 (t, 2H), 2.30 (t, 2H), 1.78 (pentet, 2H), and 1.62–1.27 (m, 4H) ppm.

Methyl 6-aminohexanoate hydrochloride. To a 1 L round bottom flask, charged with 40.0 g aminocaproic acid, was added 500 mL of methanol. Hydrogen chloride gas was bubbled through the mixture for 5 minutes, and the mixture was stirred at room temperature for 5 hours. The mixture was then concentrated via rotary evaporation and then under full vacuum pump pressure (<0.1 mm Hg) to afford 55 g of the product as a white solid (99%): ^{1}H-NMR (200 MHz, CD_3OD); 3.67 (s, 3H), 3.02 (t, 2H), 2.68 (s, 3H), 2.48 (t, 2II), and 2.03–1.87 (pentet, 2II) ppm.

Methyl 6-(trifluoroacetamido)-hexanoate: To a 1 L round bottom flask, charged with 25.0 g (138 mmol) of methyl 6-aminohexanoate hydrochloride and 500 mL of methylene chloride, was added 24 mL (170 mmol) trifluoroacetic anhydride. The mixture was cooled in an ice bath, and 42 mL (301 mmol) of triethylamine was added over a 25–30 minute period. The mixture was stirred at 0° C. to room temperature for 2 hours and then concentrated. The residue was diluted with 150 mL of diethyl ether and 150 mL of petroleum ether, and the resulting solution was washed first with 1N aqueous HCl (3×150 mL) and then with saturated aqueous sodium bicarbonate (3×150 mL). The organic phase was dried over magnesium sulfate, filtered and concentrated to give 32.9 g of the product as a pale yellow oil (99%): ^{1}H-NMR (200MHz, d_6-DMSO); 9.39 (m, 1H), 3.57 (s, 3H), 3.14 (q, 2H), 2.29 (t, 2H), 1.60–1.38 (m, 4H), and 1.32–1.19 (m, 2H) ppm.

N,N'-Bis(6-methoxycarbonylhexyl)amine hydrochloride. To a 500 mL dry round bottom flask, charged with 12.0 g (50.0 mmol) of the secondary amide, methyl 6-(trifluoroacetamido)-hexanoate, and 250 mL of dry tetrahydrofuran, was added 2.2 g (55 mmol, 1.1 equiv) of 60% sodium hydride. The mixture was stirred at room temperature for 30 minutes and then 10.25 g (49.0 mmol, 0.98 equiv) of the alkyl bromide, methyl 6-bromohexanoate, was added. The mixture was stirred at reflux for 3 hours. an additional 5.80 g (27.7 mmol, 0.55 equiv) of methyl 6-bromohexanoate was added, and the mixture was stirred at reflux for 70 hours. The mixture was cooled, diluted with 150 mL of 1N aqueous HCl and then extracted with ethyl acetate (3×100 mL). The organic extracts were combined, dried over magnesium sulfate, filtered and concentrated. The residue was diluted with 200 mL of methanol and then treated with 30 mL of 10N aqueous sodium hydroxide. The mixture was stirred at room temperature for 18 hours and then concentrated. The residue was diluted with 200 mL of deionized water and acidified to pH 1–2 with 37% concentrated HCl. The solution was washed with diethyl ether (3×100 mL). The aqueous phase was concentrated. The residue was diluted with 200 mL of methanol and reconcentrated. The subsequent residue was diluted with 250 mL of methanol, and HCl gas was bubbled through for 2–3 minutes followed by stirring at room temperature for 3 hours. The mixture was concentrated. The residue was diluted with 300 mL of methanol and filtered to remove inorganic salts. The filtrate was treated with 3 g of activated charcoal, filtered through Celite (manufactured by J. T. Baker) and concentrated. The residue, an off-white solid, was recrystallized from 100 mL of 2-propanol to afford 7.0 g of the product as a white solid. Concentration of the filtrate and further recrystallization of the residue yielded an additional 1.65 g of the product for a total of 8.65 g (56%) ^{1}H-NMR (200 MHz, d_6-DMSO); 3.57 (s, 3H), 2.90–2.73 (m, 4H), 2.30 (t, 4H), 1.67–1.44 (m, 8H), and 1.37–1.20 (m, 4H) ppm.

Methyl 4-methylaminobutyrate hydrochloride. To a 1 L round bottom flask, charged with 30.0 g (195 mmol) of 4-methylaminobutyric acid and 500 mL of methanol, was bubbled HCl gas for 1–2 minutes. The mixture was stirred at room temperature for 3–4 hours and then concentrated to afford 32.5 g of the product as a foamy, off-white solid (99%): ^{1}H-NMR (200 MHz, CD_3OD); 3.67 (s, 3H), 3.03 (t, 2H), 2.68 (s, 3H), 2.48 (t, 2H), and 2.03–1.87 (pentet, 2H) ppm.

4-Methylaminobutanol. To a 1 L round bottom flask, charged with 32.5 g (194 mmol) of the ester, methyl 4-methylaminobutyrate hydrochloride, was added 500 mL of 1M borane in tetrahydrofuran over a 1 hour period at 0° C. After the addition was complete, the mixture was refluxed for 20 hours, cooled to 0° C., and the excess borane was destroyed by careful addition of 100 mL of methanol. After

all the methanol was added, the mixture was stirred at room temperature for 1 hour and then concentrated. The residue was diluted with 400 mL of methanol and then HCl gas was bubbled into the solution for 5 minutes. The mixture was refluxed for 16 hours. The mixture was cooled, concentrated and then diluted with 250 mL of deionized water. The product was initially free based by addition of 10N aqueous sodium hydroxide, to a pH of 9–9.5, and then by addition of 70 g of AG 1 X-8 anion exchange resin (hydroxide form commercially available from BioRad), and by stirring the solution for 2 hours. The resin was filtered off and washed with 150 mL of deionized water. The aqueous filtrates were combined and concentrated. The residue was diluted with 200 mL of 2-propanol and filtered. The collected solids were rinsed with 100 mL of 2-propanol. The organic filtrates were combined and concentrated. The residue was distilled under reduced pressure to afford 12.85 g of the product as a colorless oil (bp 68° C. at 0.1–0.2 mm HG; 64%): ^{1}H-NMR (200 MIIz, D_2O); 3.52 (t, 2II), 2.56 (t, 2II), 2.31 (s, 3II), and 1.65–1.43 (m, 4H) ppm.

4-(N-Methyl-trifluoroacetamido)-1-butanol. To a 250 mL round bottom flask, charged with 10.0 g (96.9 mmol) of the amine, 4-methylaminobutanol, in 100 mL of dry methanol, was added 17.5 mL (147 mmol) of ethyl trifluoroacetate. The mixture was stirred at room temperature for 24 hours and then concentrated to afford 18.55 g of the product as a near colorless oil (96%): ^{1}H-NMR (200 MHz, D_2O); 3.63 and 3.50 (2t's, 4H), 3.20 and 3.05 (d and s, 3H), and 1.82–1.47 (m, 4H) ppm.

1-(P-Toluenesulfonyloxy)-4-(N-methyl-trifluoroacetamido)butane. To a 1 L dry round bottom, flask, charged with 17.0 g (85.4 mmol) of the alcohol, 4-(N-methyl-trifluoroacetamido-l-butanol, in 400 mL of methylene chloride, was added 17.1 g (89.7 mmol, 1.05 equiv) of toluenesulfonyl chloride followed by 30 mL (213 mmol, 2.5 equiv) of triethylamine at 0° C. over a 10 minute period. The mixture was stirred at 0° C. to room temperature for 15 hours and then washed with 5% v/v aqueous HCl (3×200 mL). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on silica gel, eluting with 50:50 hexane/methylene chloride and then with methylene chloride, to give. 25.1 g of the product as a pale yellow oil (83%): ^{1}H-NMR (200 MHz, $CDCL_3$); 7.80 (d, 2H), 7.37 (d, 2H), 4.07 (m, 2H), 3.41 (m, 3H), 3.09 and 2.98 (q and s, 3H), 2.45 (s, 3H), and 1.68 (m, 4H) ppm: TLC (methylene chloride) R_f=0.31.

1-S-(2,3,4,6-tetra-O-acetyl-beta-D-galacto-pyranosyl)-2-thiopseudourea hydrobromide. To a 250 mL round bottom flask, charged with 5.08 g (60.8 mmol, 1.09 equiv) of.thiourea and 36 mL of acetone, was added 25.0 g. (66.7 mmo9l) of tetra-acetyl-alpha-D-galactopyranosyl bromide. The mixture was stirred at reflux for 15–20 minutes and then cooled on ice. The mixture was filtered into a Buchner funnel and rinsed with 25 mL of ice cold acetone. The solids were treated with 50 mL of acetone, refluxed for 15 minutes, cooled on ice, and filtered. The solids were rinsed with 25 mL of cold acetone, air dried and then dried under vacuum to give 22.6 g of the product as a white solid (76%): ^{1}H-NMR (200MHz, d_6-DMSO); 9.4–9.0 (broad d, 4H), 5.63 (d, 1H), 5.38 (d, 1H), 5.23 (dd, 1H), 5.09 (t, 1H), 4.40 (t, 1H), 4.04 (dd, 1H), 2.13 (s, 3H), 2.08 (s, 3H), 2.00 (s, 3H), 1.93 (s, 3H) ppm.

4-(N-Methylaminobutyl)-1-thio-beta-D-galactoovranoside. To a 500 mL round bottom flask, charged with 20.7 g (42.5 mmol, 1.07 equiv) of the thiopseudourea hydrobromide prepared as described above in 70 mL of deionized water, was added 6.4 g (46.3 mmol, 1.16 equiv) of potassium carbonate and 4.7 g (45.2 mmol, 1.13 equiv) of sodium bisulfite followed immediately by 14.1 g (39.9 mmol, 1.0 eguiv) of the tosylate, 1-(p-toluenesulfonyloxy)-4-(N-methyltrifluoroacetamido)butane in 70 mL of acetone. The mixture was stirred at room-temperature for 16 hours. The mixture was diluted with 50 mL of brine and extracted with ethyl acetate (3×200 mL). The organic extracts were combined, dried over magnesium sulfate, filtered and.concentrated. The residue was chromatographed on silica gel, eluting first with 75% methylene chloride/hexane, followed by methylene chloride, then with 2% methanol/methylene chloride and finally with 10% methanol/methylene chloride. Fractions containing alkylation product with different degrees of acetylation were combined and concentrated. The residue was diluted with 250 mL of methanol and 150 mL of deionized water.and treated with 110 g of AG-1 X-8 resin (hydroxide form; 2.6 m equivig dry weight) commercially available from BioRad. The mixture was stirred at room temperature for 18 hours. The mixture was filtered, and the resin was rinsed with methanol (2×150 mL). The filtrates were combined and concentrated to afford 6.1 g of product (54%): ^{1}H-NMR (200 MHz, D_2O); 4.38 (d, 1H), 3.88 (d, 1H), 3.69–3.41 (m, 5H), 2.82–2.64 (m, 4H), 2.43 (s, 3H), and 1.68–1.57 (, 4H) ppm.

Biotin bis-methyl ester: To a 50 mL round bottom flask, charged with 1.00 g (3.23 mmol, 1.13 equiv) of amine hydrochloride, N,N'-bis-(6-methoxycarbonyl-hexyl)amine hydrochloride), and 1.30 g (2.86 mmol) of caproamidobiotin-NHS-ester (preparable by standard methods or commercially available from Sigma Chemical Company) and 10 mL of dry dimethylformamide, was added 1.5 mL (10.6 mmol) of triethylamine. The mixture was stirred at 85° C. for 2 hours and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on silica gel, eluting with 75:25:0.05 ethyl acetate/methanol/acetic acid, to afford 1.63 g of the product as a white foamy solid (93%): ^{1}H-NMR (200 MHz d_6-DMSO); 7.72 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 6H), 3.23–2.91 (m, 7H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.35–2.13 (m, 6H), 2.03 (t, 2H), 1.65–1.10 (m, 24II) ppm: TLC; R_f=0.58 (75:25:0.01 ethyl acetate/methanol/acetic acid).

Biotin bis-acid: To a 200 mL round bottom flask, charged with 1.61 g (2.63 mmol) of biotin bis-methyl ester and 50 mL of methanol, was added 5 mL of 3N aqueous sodium hydroxide. The mixture was stirred at 40° C. for 3 hours and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 50 mL of deionized water, and then 3N aqueous HCl was added until a pH of 1–2 was attained. The mixture was again concentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 20:80:–0.1 acetonitrile/water/trifluoroacetic acid and then with 50:50:0.1 acetonitrile/water/trifluoroacetic acid. The fractions containing product were combined and concentrated. The residue was diluted with 40 mL of water and 20 mL of acetonitrile. The solution was frozen (–70° C.) and lyophilized to afford 1.42 g of the product as a fluffy white solid (92%): ^{1}H-NMR (200 MHz d_6-DMSO); 7.72 (t, 1H), 6.61 (broad s, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.35–2.93 (m, 7H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.28–2.12 (m, 6H), 2.03 (t, 2H), 1.68–1.10 (m, 24H) ppm: TLC; R_f=0.30 (50:50:0.01 acetonitrile/water/trifluoroacetic acid).

Biotin tetra-methyl ester: To a 50 mL round bottom flask, charged with 350 mg (0.599 mmol) of the biotin bis-acid, 402 mg (1.30 mmol, 2.16 equiv) of amine hydrochloride, N,N'-bis-((6-methoxycarbonyl-hexyl)amine hydrochloride),

and 10 mL of dry dimethylformamide, was added 556 mg (1.26 mmol, 2.10 equiv) BOP and 500 microliters (3.54 mmol, 5.91 equiv) of triethylamine. The mixture was stirred at room temperature for 2 hours and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 85:15 methanol/water, to afford 618 mg of the product as a foamy white solid (95%): ^{1}H-NMR (200 MHz d_6-DMSO); 7.71 (t, 1H), 6.1 (broad s, 2H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 12H), 3.25–2.91 (m, 15H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.35–2.12 (m, 14H), 2.02 (t, 2H), 1.65–1.10 (m, 48H) ppm: TLC; R_f=0.48 (85:15 methanol/water).

Biotin tetra-acid: To a 50 mL round bottom flask, charged with 350 mg (0.319 mmol) of biotin tetramethyl ester and 15 mL of methanol, was added 5 mL of 1N aqueous sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 hours and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 15 mL of deionized water, acidified to pH 1–2 by addition of 6N aqueous HCl and then reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 70:30 methanol/water. The fractions containing the product were combined and concentrated. The residue was diluted with 10 mL of water and 8 mL of acetonitrile. The solution was frozen (−70° C.) and lyophilized to afford 262 mg of the product as a fluffy white solid (79%): ^{1}II-NMR (200 MHz d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.25–2.93 (m, 15H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.31–2.10 (m, 14H), 2.02 (t, 2H), 1.63–1.09 (m, 48H) ppm: TLC; R_f=0.45 (70:30 methanol/water).

Biotin octa-methyl ester: To a 25 mL round bottom flask, charged with 220 mg (0.710 mmol, 4.93 equiv) of amine hydrochloride, N,N'-bis-(6-methoxycarbonyl-hexyl)amine hydrochloride), 150 mg (0.144 mmol) of the biotin tetra-acid, and 5 mL of dry dimethylformamide, was added 300 mg (0.678 mmol, 4.71 equiv) BOP followed by 500, microliters (3.54 mmol, 24.0 equiv) of triethylamine. The mixture was stirred at room temperature for 3 hours and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 90:10 methanol/water, to afford 246 mg of the product as a foamy white solid (83%): ^{1}H-NMR (200 MHz d_6-DMSO) ;-7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 24H), 3.25–2.91 (m, 31H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.32–2.12 (m, 30H), 2.02 (t, 2H), 1.65–1.08 (m, 96H) ppm: TLC; R_f=0.42 (90:10 methanol/water).

Biotin octa-acid: To a 50 mL round bottom flask, charged with 235 mg (0.114 mmol) of biotin octa-methyl ester and 10 mL of methanol, was added 5 mL of 1N aqueous sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 hours and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 10 mL of deionized water, acidified to pH 1–2 by addition of 6N aqueous HCl and then reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 50:50 methanol/water and then with 75:25 methanol/water. The fractions containing the product were combined and concentrated. The residue was diluted with 20 mL of 1:1 (ratio by volume) acetonitrile/water. The solution was frozen (−70° C.) and lyophilized to afford 202 mg of the product as a fluffy white solid (91%): 1H-NMR (200 MHz d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.29–2.91 (m, 31H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.31–2.10 (m, 30H), 2.03 (t, 2H), 1.65–1.09 (m, 96H) ppm: TLC; R_f=0.51 (75:25 methanol/water).

Biotin hexadeca-methyl ester: To a 25 mL round bottom flask, charged with 154 mg (0.497 mmol, 10.0 equiv) of amine hydrochloride, N,N'-bis-(6methoxycarbonyl-hexyl) amine hydrochloride), 97 mg (0.0497 mmol) of the biotin octa-acid, and 5 mL of dry dimethylformamide, was added 202 mgr (0.457 mmol, 9.2 equiv) BOP followed by 500 microliters (3.54 mmol, 71.2 equiv) of triethylamine. The mixture was stirred at room temperature for 8 hours and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on silica gel, eluting first with 70:30 methanol/water and then with 95:5 methanol/water; to afford 149 mg of the product as a foamy white solid (75%): ^{1}H-NMR (200 MHz d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.57 (s, 48H), 3.25–2.92 (m, 63H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.35–2.11 (m, 62H), 2.01 (t, 2H), 1.65–1.08 (m, 192H) ppm: TLC; R_f=0.31 (95:5 methanol/water).

Biotin hexadecyl-acid: To a 50 mL round bottom flask, charged with 141 mg (0.0353 mmol) of biotin hexadeca-methyl ester and 15 mL of methanol, was added 8 mL of IN aqueous sodium hydroxide and 5 mL of deionized water. The mixture was stirred at room temperature for 14 hours and then concentrated via reduced pressure rotary evaporation. The residue was diluted with 15 mL of deionized water, acidified to pH 1–2 by addition of 6N aqueous HCl and then reconcentrated. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 85:15 methanol/water. The fractions containing the product were combined and concentrated. The residue was diluted with 20 mL of 1:1 acetonitrile/water. The solution was frozen (−70° C.) and lyophilized to afford 130 mg of the product as a fluffy white solid (75%): H-NMR (200 MHz d_6-DMSO); 7.71 (t, 1H), 6.41 (s, 1H), 6.34 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.26–2.92 (m, 63H) 2.81 (dd, 1H), 2.55 (d, 1H), 2.35–2.10 (m, 62H), 2.01 (t, 2H), 1.65–1.09 (m, 192H) ppm: TLC; R_f=0.64 (85:15 methanol/water).

Hexadeca-galactosyl biotin:. To a 25 mL round bottom flask, charged with 125 mg (0.0332 mmol) of biotin hexadeca-acid, 179 mg (0.636 mmol, 19.2 equiv) of galactose-amine, 4-(N-methylaminobutyl)-1-thio-beta-D-galactopyranoside, and 4 mL of dry methylformamide, was added 264 mg (0.597 mmol, 18.0 equiv) of BOP followed by 400 microliters (2.87 mmol, 86.5 equiv) of dry triethylamine. The mixture was stirred at room temperature for 17 hours and then concentrated via reduced pressure rotary evaporation. The residue was chromatographed on C-18 reverse phase silica gel, eluting first with 60:40 methanol/water and then with 75:25 methanol/water. The fractions containing the product were combined and concentrated and rechromatographed on C-18 reverse phase silica. gel, eluting first with 40:60:0.1 acetonitrile/water/trifluoroacetic acid and then with 50:50:0.1 acetonitrile/water/trifluoroacetic

acid. The fractions containing the product were again combined and concentrated. The residue was dissolved in 20 mL of water. The solution was frozen (−70° C.) and lyophilized to afford 173 mg of the product as a fluffy white solid (75%): ^{1}H-NMR (200 MHz D_2O); 4.52 (m, 1H), 4.37 (d, 15H), 3.90 (d, 16H), 3.70–3.42 (m, 80H), 3.41–3.05 (m, 97H), 2.98–2.82 (2s and 2m, 49H), 2.80–2.49 (m, 33H), 2.44–2.11 (m, 62H), 1.75–1.10 (m, 256H) ppm: TLC; R_f=0.53 (75:25 methanol /water).

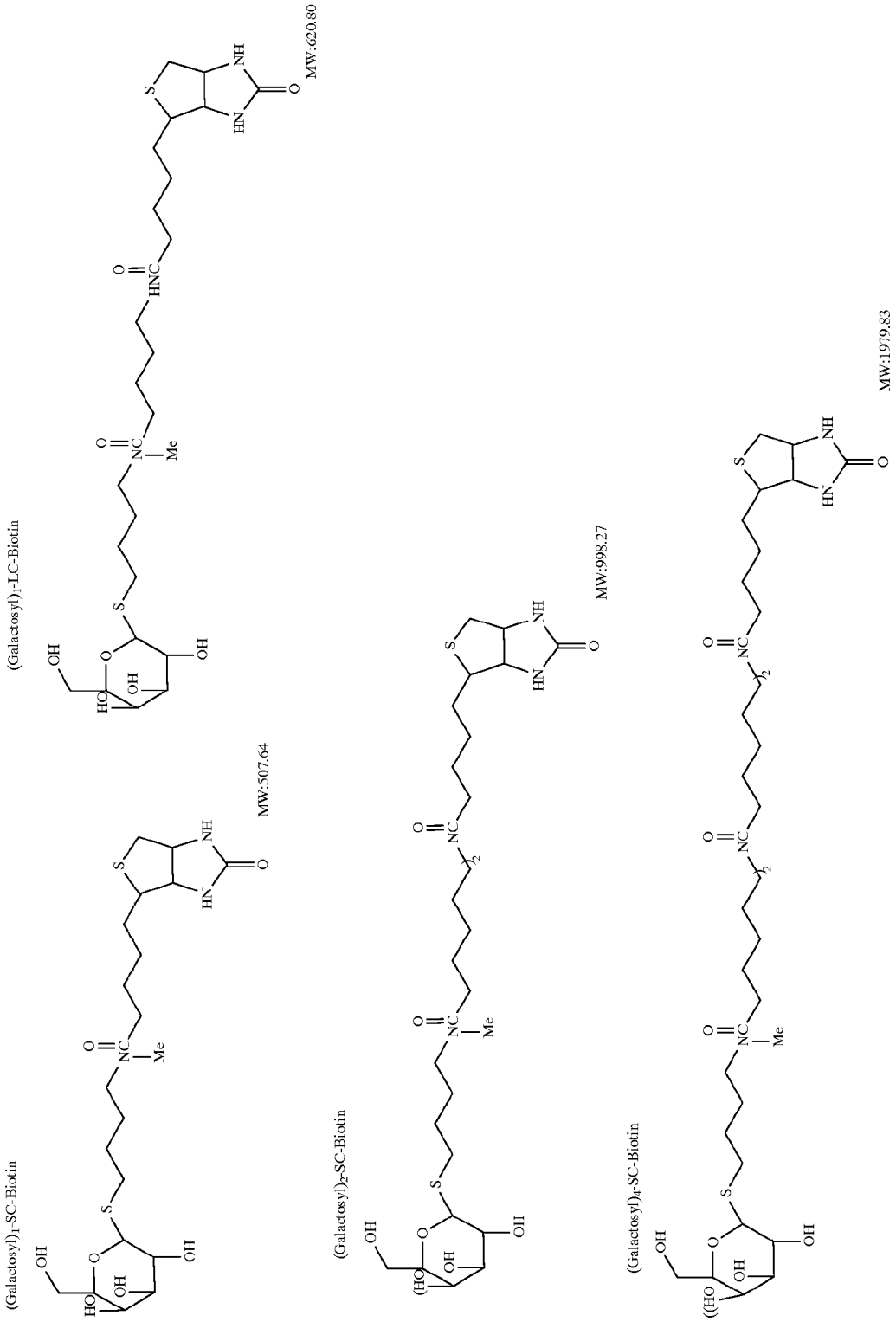
The above procedure is designed for the formation of a galactose cluster of 16 galactose residues. The four or eight galactose versions can be made in accordance with this procedure by proceeding from the tetra acid or the octa acid to the galactose derivatization step, which was described above for the 9 16-galactose cluster. Similarly, 32, etc. galactose cluster constructs can be prepared in accordance with the present invention by introduction of more iterations of the methyl ester and acid formation steps. When the desired number of acid residues are formed, the galactose derivatization step is employed, with the proportions of the components adjusted to accommodate the number of acid residues.

EXAMPLE VI

Small Molecule Clearing Agent Evaluation

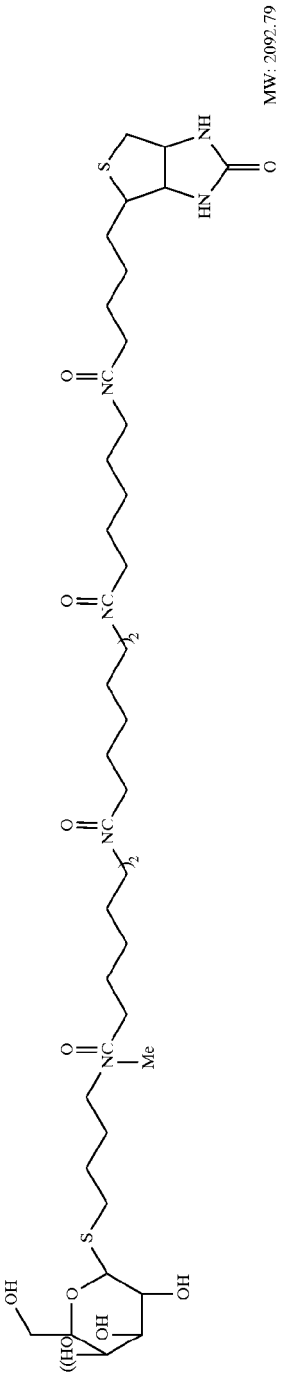
In order to demonstrate the efficacy of the described small molecule clearing agents, a number of such conjugates were synthesized using a biotin binding moiety and galactose residue cluster directors. These conjugates were synthesized using different numbers of attached galactose residues. In addition, these conjugates contained either the long chain linker (LC=containing an aminocaproyl spacer between the amine associated with galactose and the carboxyl moiety associated with the biotin) or the short chain linker (SC= direct link between the amine associated with galactose and the carboxyl moiety associated with the biotin) as set forth below.

The conjugates involved in the testing are depicted below:

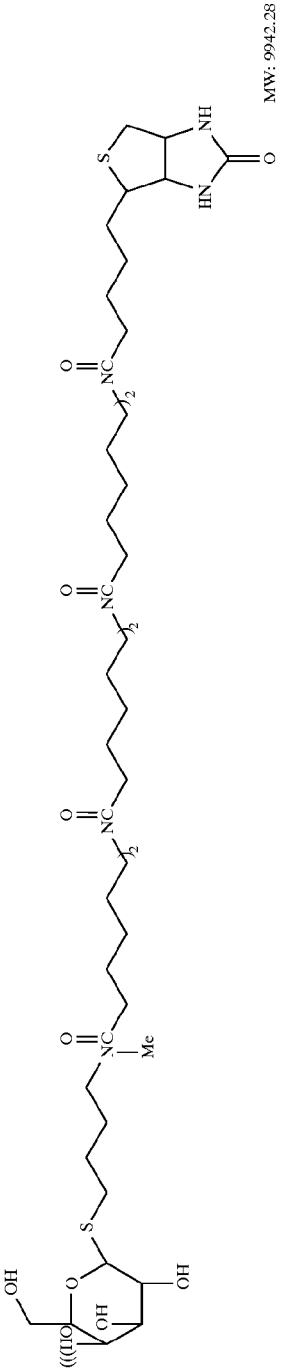

(Galactosyl)1-SC-Biotin
MW:507.64
(Galactosyl)2-SC-Biotin
MW:998.27
(Galactosyl)4-SC-Biotin
MW:1979.83
(Galactosyl)1-LC-Biotin
MW:620.80

-continued

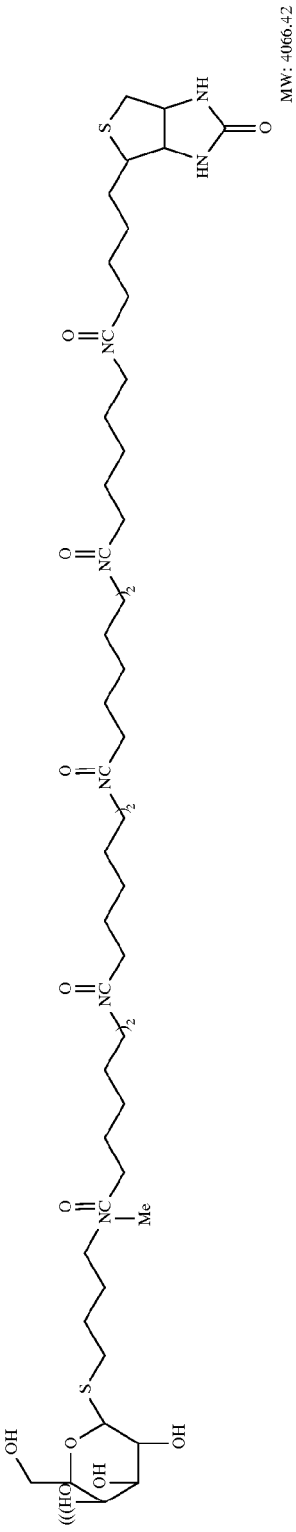
(Galactosyl)$_1$-LC-Biotin



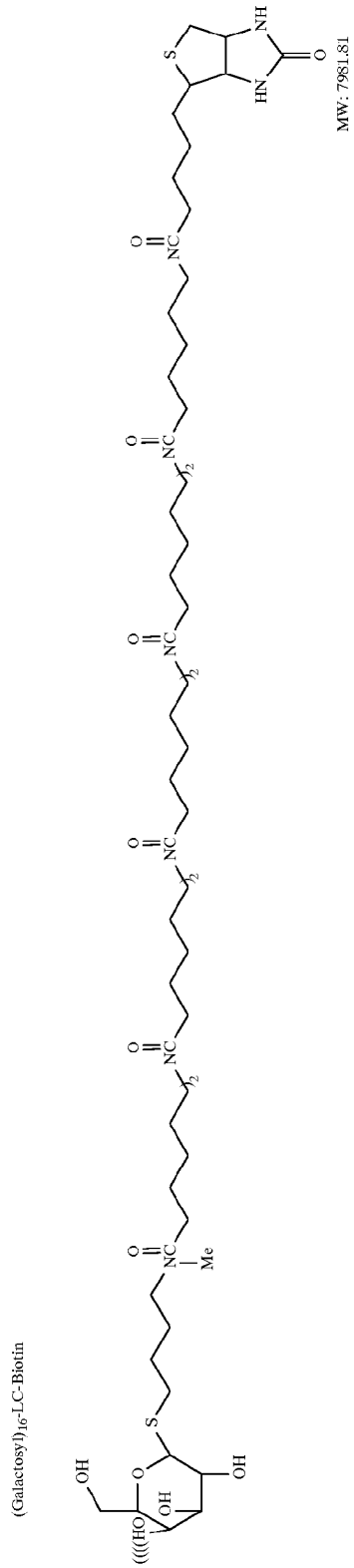
(Galactosyl)$_5$-SC-Biotin



(Galactosyl)$_9$-LC-Biotin



-continued



Some or all of these compounds were assayed for their clearance directing activity in two sets of experiments. The first set of experiments involved ex vivo preparation of a precomplexed monoclonal antibody-streptavidin-biotin-galactose cluster conjugate labeled with I-125, intravenous administration of the conjugate in a mouse model, and measuring serum levels of the conjugate over time. The second set of experiments involved intravenous administration of MAb-streptavidin conjugate followed by administration of biotin-galactose cluster conjugate.

NR-LU-10 antibody (MW 150 kD) was conjugated to streptavidin (MW 66 kD) (as described in Example II above), and radiolabeled with ^{125}I/PIP-NHS as described below. The antibody component of the conjugate was radioiodinated using p-aryltin phenylate NHS ester (PIP-NHS) and ^{125}I sodium iodide. In general, the experimentation involving the 2, 4 and 8 galactose-biotin constructs was conducted in an analogous manner to that for the 16 galactose-biotin construct as described below.

The data from these experiments indicates that no significant increase in serum clearance (in comparison to the MAb-Streptavidin conjugate itself) occurs until at least 4 galactose residues are attached to the biotin molecule. In addition, the data indicates that the longer linker separating the galactose cluster from the biotin molecule resulted in better clearance rates. This is consistent with the inventors' belief that the galactose cluster interferes with binding to the conjugate to be cleared if an appropriate length spacer is not used to minimize steric interactions or that sugar-hepatocyte interaction is sterically precluded.

In a third set of experiments conducted in vivo in the pretargeting format (e.g., administration of radiolabeled MAb-streptavidin conjugate followed by administration of clearing agent), the $(galactosyl)_8$-LC-biotin conjugate was also compared to galactose-HSA-biotin prepared as described above. This comparison was conducted in a Balb/c mouse model and was for the ability to clear an I-125 labeled monoclonal antibody-streptavidin conjugate (1–125 LU-10-streptavidin) from circulation as a function of time. The results of this experiment indicate that the $(galactosyl)_8$-LC-biotin conjugate is comparable to galactosylated-HSA-biotin in its ability to clear the streptavidin-containing conjugate from circulation. Subsequent experiments have further shown that hepatic-directed compounds containing 16 galactose residues provide for even better clearance than those containing 8 galactose residues.

Experiments were designed and executed to evaluate a 16 galactose cluster-biotin construct without the stabilizing tertiary amine structure of the nitrogen of the amide closest to the biotin, the preparation of such a stabilized construct being described above in Example V. BALB/c female mice (20–25 g) were injected i.v. with 120 micrograms of NR-LU-10-streptavidin conjugate radiolabeled with I-125, and blood was serially collected from n=3 mice. The clearance of the conjugate from the blood was measured of these control mice. Separate groups of mice were injected with either 120 or 12 micrograms of radiolabeled monoclonal antibody-streptavidin conjugate which had been precomplexed with the 16 galactose-biotin construct by mixing the biotin analog at a 20-fold molar excess with the antibody conjugate, and purifying the excess small molecule from the protein by size exclusion chromatography. Both doses of precomplexed conjugate showed extremely rapid clearance from the blood, relative to the antibody conjugate control.

Having shown that precomplexed material could clear rapidly and efficiently from the blood, experiments were conducted to measure the effectiveness of various doses of-the 16 galactose-biotin construct to form rapidly clearing complexes in vivo. Mice received 400 micrograms of I-125 NR-LU-10streptavidin conjugate intravenously, and approximately 22 hours later received the 16 galactose-biotin construct at doses of 100 50, or 10:1 (456, 228 and 45 micrograms, respectively) molar excess to circulating monoclonal antibody-streptavidin conjugate. While each dose was effective at clearing conjugate, the most effective dose (both kinetic and absolute) was the 10:1 dose. For the larger doses, there appears to be some saturation of the liver receptor, since both larger doses show a plateau in conjugate clearance for about 1 hour after administration of the 16 galactose-biotin construct. The larger doses may be sufficiently high to achieve competition between complexed and non-complexed 16-galactose-biotin for liver receptors, thereby precluding all but a small initial fraction of the complexed MAb-streptavidin conjugate from clearing via the liver. Following the plateau period, clearing of the conjugate remained slow and was eventually less complete than that achieve with the lower dose (approximately 10% of the conjugate remained in circulation at the higher doses, in comparison to 2% for the lower dose). An alternative explanation for this finding rests on the fact that the 16-galactose-biotin construct was not stabilized to potential biotinidase-mediated cleavage (e.g., the chemical synthesis did not incorporate a methyl, lower alkyl, carboxylic acid, lower alkyl carboxylic acid or like group was not bound to the amide nitrogen most closely adjacent the biotin rather than hydrogen). If the 16 galactose-biotin construct is unstable, sufficient biotin may be released at higher doses to that a significant portion of circulating conjugate became blocked thereby and, consequently, was not cleared via hepatic-mediated uptake.

Evident in all groups is the lack of a "rebound" or gradual increase in blood levels of circulating conjugate following disruption of the equilibrium between vascular and extravascular concentrations of conjugate. This constitutes the best evidence to date that galactose cluster-biotin constructs extravasate into extravascular fluid, and that conjugate which is. complexed extravascularly clears very rapidly when it passes back into the vascular compartment.

Further experimentation in the same animal model compared $(galactose)_{35}$-HSA-$(biotin)_2$ clearing agents prepared as described above and decreasing doses of 16 galactose-biotin construct as in vivo clearing agents. A 46 microgram dose of 16 galactose-biotin was found to be optimal and more effective than the previously optimized dose of $(galactose)_{35}$-HSA-$(biotin)_2$. Lower (12 and 23 microgram) and higher (228 microgram) doses of 16 galactose-biotin were less efficient at removing circulating conjugate, and the lower doses showed a significant rebound effect, indicating that incomplete complexation with circulating conjugate may have occurred.

Having shown that effective clearing could be achieved with the appropriate does of 16 galactose-biotin construct, studies were undertaken in tumored nude mice to evaluate the potential blockade of tumor-associated conjugate by the small 16 galactose-biotin. Mice bearing either SW-1222 colon tumor xenografts or SHT-1 small cell lung cancer (SCLC) tumor xenografts were pretargeted with NR-LU-10-streptavidin conjugate and, 22 hours later, received 46 micrograms of 16 galactose-biotin. After 2 hours, Y-90-DOTA-biotin prepared as described above was administered, and its uptake and retention in tumor and non-target tissues was evaluated by sacrifice and tissue counting for radioactivity 2 hours post-administration.

In comparison to historical controls employing (galactose)$_{35}$-HSA-(biotin)$_2$, tumor targeting was slightly lower in the high antigen-expressing colon xenograft and was slightly higher in the low antigen-expressing SCLC xenograft. Given the normal variability in such experiments, tumor uptake of radioactivity was assessed as roughly equivalent, a surprising result given the potential for target uptake of 16 galactose-biotin. Non-target organ uptake was comparable in all tissues except liver, where animals receiving 16 galactose-biotin showed slightly higher levels. The historical controls were conducted with a 3 hour time period between clearing agent and radioactivity administration. When such a 3 hour period was allowed between 16 galactose-biotin and radioactivity administration, the liver levels. were lower and comparable to that of the HSA-containing agent (approximately 1% injected dose/gram).

Experiments were. also carried out using I-125 labeled MAb-streptavidin conjugate and In-111 labeled DOTA-biotin to assess the relative stoichiometry of those materials at the tumor target site using 16 galactose-biotin as a clearing agent. Previous studies with (galactose)$_{35}$-HSA-(biotin)$_2$ had shown that an expected 4:1 ratio of DOTA-biotin to MAb-streptavidin (streptavidin has 4 biotin binding sites) could be achieved at the tumor with an optimized dose of that clearing agent. When a similar protocol was employed with the 16 galactose-biotin construct, the ratio of DOTA-biotin to MAb-streptavidin was only 2.65. This indicated that some filling of tumor-associated streptavidin may have occurred, although the nature of such blockage (16 galactose-biotin or biotin released therefrom) was undetermined. Experiments to assess the nature of this blockade are underway.

In summary, galactose cluster conjugates exhibited ability to clear circulating conjugate, provided the galactose cluster contains a sufficient number of appropriately spaced galactosyl residues. 16 Galactose-biotin has proven to be an effective construct for clearing MAb-streptavidin from the circulation (both vascular and extravascular spaces). Despite an apparent blockade of some pretargeted biotin binding sites at the tumor, efficient tumor targeting can still be achieved using this agent. Stabilization of the linkage between biotin and the galactose cluster may minimize any tumor-associated biotin binding site compromise by the galactose cluster-biotin construct.

EXAMPLE VII

Director Reagent Preparation

This procedure is schematically shown in FIG. **3**.

N-BOC-Bis-methylester. To 1.00 g (3.23 mmol) of the amine hydrochloride, N,N-bis-(6-methoxycarbonyl-hexyl) amine hydrochloride prepared as described above, was added 1.5 mL (10.6 mmol) of triethylamine followed by 875 mg (3.55 mmol, 1.1 equiv) of BOC-ON, 2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile. The mixture was stirred at room temperature for 18 hours and then concentrated. The residue was diluted with 100 mL of ethyl acetate and washed with 1N aqueous hydrochloric acid (3×50 mL), followed by saturated aqueous sodium bicarbonate (2×mL). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on silica gel, eluting with 15% (percentage based upon volume) ethyl acetate/hexane. Chromatographic fractions containing product were combined and concentrated to afford 990 mg of product as a near colorless oil (83%).

N-BOC-Bis-acid. To 980 mg (2.62 mmol) of the diester prepared in the previous step in 10 mL of methanol was added 5.8 mL of 1N aqueous sodium hydroxide (5.8 mmol). The mixture was stirred at room temperature for 16 hours and then concentrated. The residue was diluted with 30 mL of deionized water and acidified to pH 1.5–2. The mixture was extracted with ethyl acetate (6×50 mL). The organic extracts were dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on reverse phase C-18 silica gel commercially available from J. T. Baker, eluting with 65% methanol/water. Chromatographic fractions containing product were combined and concentrated to afford 851 mg of product as a near colorless oil (94%).

N-BOC-Tetra-methyl ester. To 825 mg (2.39 mmol) of the bis-acid prepared as described above in 35 mL of dry dimethylformamide, was added 1.75 g (5.65 mmol, 2.36 equiv) of amine hydrochloride, N,N-bis-(6-methoxycarbonylhexyl)amine hydrochloride, and 3.0 mL of triethylamine followed by 2.4 g (5.4 mmol, 2.3 equiv) of BOP. The mixture was stirred at room temperature for 17 hours and then concentrated. The residue was diluted with 100 mL of ethyl acetate and washed with 1N hydrochloric acid (3×50 mL) followed by washing with aqueous sodium bicarbonate (2×50 mL). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on silica gel, eluting with ethyl acetate. Chromatographic fractions containing product were combined and concentrated to afford 1.63 g of the product as a near colorless oil (80%).

N-BOC-Tetra-acid. To a solution of 1.41 g (1.65 mmol) of tetra-methyl ester prepared as described above in 25 mL of methanol was added 7.4 mL (7.4 mmol) of 1N aqueous sodium hydroxide. The mixture was stirred at room temperature for 22 hours and then concentrated. The residue was diluted with 30 mL of deionized water and acidified to pH 2 with 1N aqueous hydrochloric acid. The mixture was extracted with 3:1 (ratio by volume) ethyl acetate/isopropanol (3×100 mL). The organic extracts were concentrated. The residue was chromatographed on reverse phase C-18 silica gel, eluting initially with 50:50 (ratio by volume) methanol/water and eventually with 75:25 methanol/water. Chromatographic fractions containing product were combined and concentrated to afford 1.19 g of the product as a colorless oil (90%).

N-BOC Octa-methyl ester. To a mixture of 501 mg (0.626 mmol) of tetra-acid prepared as described above and 30 mL of dry dimethylformamide was added 968 mg (3.12 mmol, 5.0 equiv) of amine hydrochloride, N,N'-bis-(6-methoxycarboxyhexyl)amine hydrochloride, and 2.0 mL (14.2 mmol) of triethylamine, followed by 1.22 g (2.76 mmol, 4.6 equiv) BOP. The mixture was stirred at room temperature for 19 hours and then concentrated. The residue was diluted with 75 mL of ethyl acetate and washed with 1N aqueous hydrochloric acid (2×50 mL). The organic.phase was dried over magnesium sulfate, filtered and concentrated. The residue was chromatographed on reverse phase C-18 silica gel, eluting initially with 60:40 methanol/water and eventually with 90:10 methanol/water. The chromatographic fractions containing product were combined and concentrated to afford 715 mg of the product as a colorless oil (63%).

N-BOC Octa-acid. To a solution of 715 mg (0.393 mmol) of octa-methyl ester prepared as described above in 20 mL of methanol was added 6 mL of 1N aqueous sodium hydroxide (6 mmol) and 5 mL of deionized water. The mixture was stirred at room temperature for 16 hours and

then concentrated. The residue was diluted with 20 mL of deionized water, and the solution was acidified to pH 1.5–2.0. The mixture was concentrated, and the residue was chromatographed on reverse phase C-18 silica gel, eluting initially with 50:50 methanol/water and eventually with 80:20 methanol/water. The chromatographic fractions containing product were combined and concentrated to afford 647 mg of the product as a near colorless oil (96%).

The above procedure is designed for the formation of a galactose cluster of 8 galactose residues. The four galactose version could be made in accordance with this procedure by proceeding from the tetra acid to the galactose derivatization step, which is described below for the 8-galactose cluster. Similarly, 16, 32, etc. galactose cluster constructs can be prepared in accordance with the present invention by introduction of two more iterations of the methyl ester and acid formation steps. More specifically, the 16-methyl ester construct, the 16-acid, the 32-methyl ester and so on would be prepared essentially as described above for the tetra and octa forms. When the desired number of acid residues are formed, the galactose derivatization step is employed, with the proportions of the components adjusted to accommodate the number of acid residues.

N-BOC-Octa-galactosyl construct. To a mixture of 161 mg (94 mmol) of octa-acid prepared as described above and 225 mg (906 micromol, 9.64 equiv) of galactose amine, 4-N-methylaminobutyl-1-thio-beta-D-galactopyranoside, in 8 mL of dry dimethylformamide was added 0.5 mL (3.54 mmol) of triethylamine followed by 371 mg (839 micromol, 8.4 equiv) of BOP. The mixture was stirred at room temperature for 17 hours and then concentrated. The residue was chromatographed on reverse phase C-18 silica gel, eluting initially with 40:60 methanol/water and finally with 70:30 methanol/water. The chromatographic fractions containing product were combined and concentrated to afford 170 mg of the product as a near colorless oil (47%).

Octa-galactosyl amine. To 170 mg of the N-BOC-octa-galactosyl construct prepared as described above was added 5 mL of trifluoroacetic acid. The mixture was stirred at room temperature for 10 minutes and then concentrated. The residue was diluted with 10 mL of methanol and reconcentrated. The residue is used without further purification.

Other director reagent families bearing functional groups other than the amine group of the construct formed above can be made from the amine construct using standard chemical techniques conversion of amines to other functional groups.

EXAMPLE VIII

Extended Director Reagent Preparation

Extender-Galactose Cluster Preparation.

This procedure is schematically shown in FIG. **4**. This-procedure is undertaken, if necessary, to facilitate director reagent conjugation. The extension procedure, which in this example preserves an amine functional group, can also be used to introduce an alternative functional group as discussed herein.

Methyl 6-(N-BOC)-aminocaoroate. To a mixture of amine hydrochloride, methyl-6-aminohexanoate hydrochloride, prepared as described above is added 1.1 equivalents of BOC-ON followed by 2–3 equivalents of triethylamine. The mixture is stirred at 15–30° C. for 16–24 hours and the concentrated. The residue is dissolved in ethyl acetate and washed with 1N aqueous hydrochloric acid and then with saturated aqueous sodium bicarbonate. The organic phase is dried over magnesium sulfate, filtered and concentrated via reduced pressure rotary evaporation. The residue is chromatographed on silica gel, eluting with 25% ethyl acetate/hexane. The chromatographic fractions containing the product are combined and concentrated to afford the product.

6-(N-BOC)-aminocaproic acid. To a solution of the methyl ester, methyl 6-(N-BOC)-aminocaproate, in methanol is added 1.5 equivalents of 1N aqueous sodium hydroxide. The mixture is stirred at 15–30° C. for 16–24 hours and then concentrated. The residue is diluted with deionized water and extracted with ethyl acetate. The organic extracts are combined, dried over magnesium sulfate, filtered and concentrated. The residue is chromatographed on silica gel, eluting initially with 25% ethyl acetate/hexane and finally with 100% ethyl acetate. The chromatographic fractions containing the product are combined and concentrated to afford the product.

N-BOC extended octa-galactosyl construct. To a solution of the octa-galactosyl amine prepared as described above in dimethylformamide and 1.5–3 equivalents of 6-(N-BOC)-aminocaproic acid is added 4–6 equivalents of triethylamine followed by 1.1–1.5 equivalents of BOP. The mixture is stirred at 15–30° C. for 4–24 hours and then concentrated. The residue is diluted with deionized water, and the pH is adjusted to 1.5–2.0 by addition of 1N aqueous hydrochloric acid. The mixture is washed with ethyl acetate. The aqueous phase is concentrated, and the residue is chromatographed on reverse phase C-18 silica gel, eluting initially with 50:50 methanol/water and finally with 65:35 methanol/water. The chromatographic fractions containing product are combined and concentrated to afford the product.

Amine extended octa-galactosyl construct. To the N-BOC protected amine prepared in the previous step is added trifluoroacetic acid. The mixture is stirred at 15–30° C. for 10 minutes and then concentrated. The residue is diluted with methanol and reconcentrated to afford the product which is used without further purification.

EXAMPLE IX

Radiolabeled Annexin-Galactose Cluster Conjugates Trifunctional Linker Approach

A. Chelate Preparation.

Production of chelate N,N'-bis(2-disulfidyl-4-methylphenyl)-gamma,gamma'-diamino-isovalerate N-hydroxysuccinimide, as shown schematically in FIG. **5**.

3-Iodomethyl-4-iodobutyric acid: To a solution of 1.61 g (10 mmole) 3-hydroxymethyl-4-butanolide (prepared by the procedure of Kinoshita and Hirano, *J. Hetrocyclic Chem.*, 29: 1025, 1992) in 100 mL carbon tetrachloride is added 8 g (40 mmole) of iodotrimethylsilane. The reaction mixture is heated at 50° C. for 12 hours under nitrogen. The mixture is diluted with chloroform and washed with water (3×100 mL), 5% aqueous sodium thiosulfate (100 mL), 10% aqueous sodium bicarbonate and brine. The organic layer is dried over magnesium sulfate, filtered and evaporated to give the desired crude product. The crude product is purified by silica gel chromatography (ethyl acetate-hexane=3:7 as the eluting solvent) to give 3-iodomethyl-4-iodobutyric acid.

Ethyl-3-iodomethyl-4-iodobutyrate: A solution of 2.831 g (8 mmole) 3-iodomethyl-4-iodobutyric acid in 80 mL ethanol is saturated with HCl gas at 0° C. After stirring the solution at room temperature for two days, the solvent is removed under vacuum, and the residue is dissolved in dichloromethane. The dichloromethane layer is washed with 10% aqueous sodium bicarbonate (3×100 mL), water (1×100 mL) and brine. The separated dichloromethane layer is dried over with magnesium sulfate, filtered and evaporated to give ethyl-3-iodomethyl-4-iodobutyrate.

Ethyl-qamma, gamma'-di(4-methylanilino) isovalerate: A stirred solution of 7.5 g (70 mmole) 4-toluidine, 2.764 g (7 mmole) ethyl-3-iodomethyl-4-iodobutyrate and 0.588 g (7 mmole) sodium bicarbonate in 30 mL dry dimethyl sulfoxide is heated at 100° C. for 3 hours under nitrogen. The cooled mixture is poured onto 400 mL ice water with stirring. The resulting precipitate is collected by filtration.

The remaining 4-toluidine in the precipitate is removed by washing with aqueous acetic acid several times. The product is obtained by recrystallization of the washed precipitate in heptane.

Ethyl-gamma,gamma'-[1,3-di(2-imino-6-methyl benzthiazolyl-3)]isovalerate: To a magnetically stirred suspension of 2.0 g (6.5 mmole) ethyl-gamma,gamma'-di(4-methylanilino)isovalerate in 250 mL glacial acetic acid is added ammonium thiocyanate (3.5 g, 0.046 mole) followed by the dropwise addition of a solution of bromine (7.27 g, 0.046 mole) in 50 mL glacial acetic acid. After addition is complete, stirring is continued overnight. The yellow precipitate of dihydrobromide salt is filtered and dried. The dried solid is then dissolved in hot water and the benzothiazole free base is liberated with saturated sodium bicarbonate solution. The white solid is filtered and dried to give crude product which is used without further purification.

N,N'-Bis(2-disulfidyl-4-methylphenyl)-gamma, gamma'-diaminoisovaleric aid: To a suspension of ethyl-gamma, gamma'-[1,3-di(2-imino-6-methyl benzthiazolyl-3)] isovalerate in 40 mL distilled water, solid potassium hydroxide pellets (20.0 g, 0.037 mole) are added, and the resulting solution is heated at 120° C. for 15–24 hours. After several hours of heating, the suspension becomes a clear solution. The reaction mixture is cooled in an ice bath and acidified with 5.0N acetic acid to pH 5.0, and the aqueous solution is extracted with three 100 mL portions of ethyl acetate. The combined ethyl acetate extracts are dried over anhydrous sodium sulfate and filtered. Solvent from the filtrate is removed under reduced pressure to give crude product. This crude product is chromatographed on silica gel column using a 20:80 mixture of ethyl acetate:hexane with 1% acetic acid as eluting solvent to give the product as a crystalline yellow solid.

N,N'-Bis(2-disulfidyl-4-methylphenyl)-gamma, gamma'-diaminosiovalerate N-hydroxysuccinimide: N,N'-Bis(2-disulfidyl-4-methylphenyl)-gamma,gamma'-diamino-isovaleric acid is reacted with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in either tetrahydrofuran (THF) or dimethylformamide (DMF) at room temperature. After stirring overnight at room temperature, the solvent is removed, and the crude product is purified by column chromatography on silica gel.

B. Conjugate Formation.

This chelate is amenable to use with a suitable trifunctional linker to form a radiolabeled annexin-galactose cluster conjugate of the present invention as described below.

Commercially available N-epsilon-t-BOC-lysine (Sigma Chemical Company) is converted, using trifluoroacetic anhydride, to its N-alpha-trifluoroacetamide adduct. Activation of the carboxylic acid functionality, for example with BOP (benzotriazol-1-yloxy-tris(dimethyl-amino)-phosphonium hexafluorophosphate) commercially available from Aldrich Chemical Company, and reaction of the activated moiety with the single available amine on a galactose cluster, e.g., formed as described above, affords a galactose cluster-trifunctional linker species. The alpha-amine of lysine trifunctional linker component of the galactose clustertrifunctional linker species is deblocked using methanolic sodium hydroxide. Reaction with the N-hydroxysuccinimide ester of the chelate molecule formed as set forth in part A of this example affords a galactose cluster-chelate-trifunctional linker species. Deprotection of the epsilon amine of the lysine trifunctional linker component using trifluoroacetic acid, followed by reaction with succinic anhydride provides an available carboxylic acid functionality through which the annexin may be conjugated following activation of the carboxylic acid (e.cr, with BOP).

Kits containing one or more of the components described above are also contemplated. For instance, galactose cluster-biotin conjugate may be provided in a sterile container for use in pretargeting procedures. Alternatively, such a galactose cluster-biotin conjugate may be vialed in a non-sterile condition for use as a research reagent.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

What is claimed is:

1. An improved method of therapy which comprises the administration of a compound comprising a binding moiety, targeting moiety or active agent, which is selectively delivered to a target sites, wherein the improvement comprises the addition of a reagent which provides for hepatic-directed clearance, wherein the reagent includes:

a hexose cluster characterized by at least nine hexose residues connected in an iteratively branched configuration, each branch of the configuration having three prongs, and

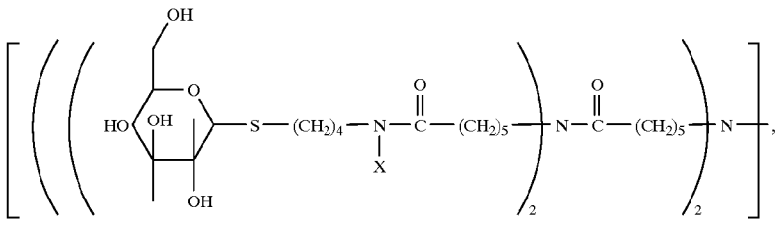
a functional moiety through which the binding moiety, targeting moiety or active agent is directly or indirectly bound.

2. The method of claim **1**, wherein the hexose cluster is formed of N-acetylgalactosamine residues.

3. The method of claim **1**, wherein the functional moiety is selected from the group consisting of: activated esters, maleimides, isocyanates, alkyl halides, hydrazides, thiols, imidates, and aldehydes.

4. An improved method of therapy which comprises the administration of a compound comprising a binding moiety, targeting moiety or active agent, which is selectively delivered to a target site, wherein the improvement comprises the addition of a reagent which provides for hepatic clearance, wherein the reagent includes:

(a) a galactose cluster having a formula selected from the group consisting of



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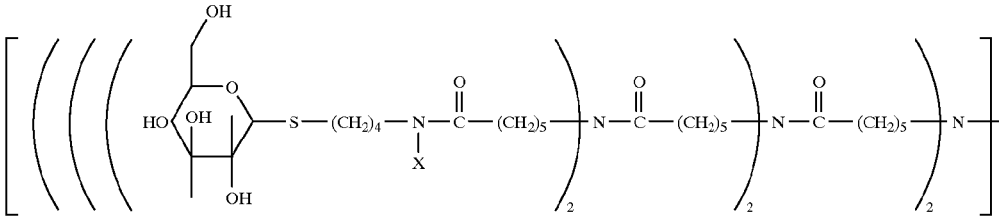
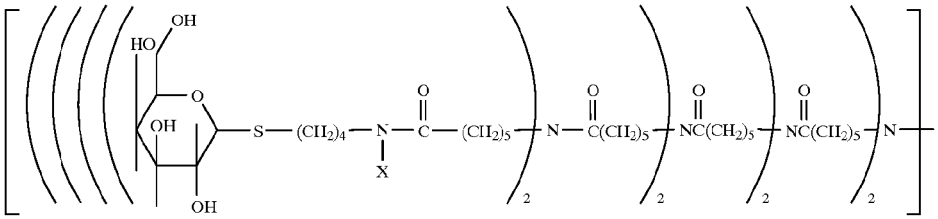
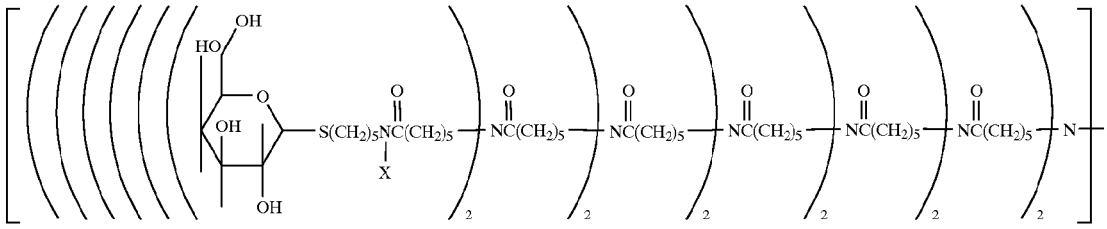




and

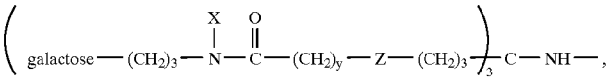


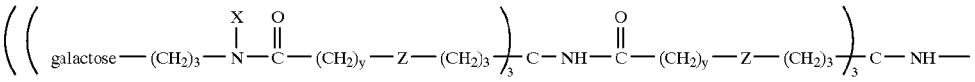
wherein X is H or methyl; and

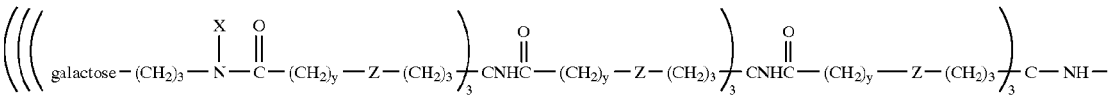
(b) a functional moiety through which the binding moiety, targeting moiety or active agent is directly or indirectly bound.

5. An improved method of therapy which comprises the administration of a compound comprising a binding moiety, targeting moiety or active agent, which is selectively delivered to a target site, wherein the improvement comprises the addition of a reagent which provides for hepatic clearance, wherein the reagent includes:

(a) a galactose cluster having a formula selected from the group consisting of







wherein X is H or methyl, y is from 1 to 10, and Z is O or S; and

(b) a functional moiety through which the binding moiety, targeting moiety or active agent is directly or indirectly bound.

* * * * *

EXHIBIT 37

From: Milind Deshpande <mdeshpande@racap.com>
To: Spiegel, David
Sent: 6/17/2019 11:36:40 PM
Subject: Re: Patent filings
Attachments: US 5985826.pdf

David,
Thanks for taking the time to meet over dinner and very much appreciate your patience. [redacted]
Milind

On Mon, Jun 17, 2019 at 9:20 PM Spiegel, David <david.spiegel@yale.edu> wrote:

Hi Milind, Great seeing you tonight and thanks again for dinner! [redacted]

[redacted]

Best,

David

--

David A. Spiegel, Ph.D., M.D.

Yale University

Professor of Chemistry and Pharmacology

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EXHIBIT 38

UNITED STATES

SECURITIES AND EXCHANGE COMMISSION

WASHINGTON, DC 20549

FORM 10-K

(Mark One)

☒ ANNUAL REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934

For the fiscal year ended December 31, 2021

or

☐ TRANSITION REPORT PURSUANT TO SECTION 13 OR 15(d) OF THE SECURITIES EXCHANGE ACT OF 1934

For the transition period from _____ to _____

Commission File Number: 001-38080

Biohaven Pharmaceutical Holding Company Ltd.

(Exact Name of Registrant as Specified in its Charter)

British Virgin Islands	Not applicable
(State or other jurisdiction of incorporation or organization)	(I.R.S. Employer Identification No.)
c/o Biohaven Pharmaceuticals, Inc. 215 Church Street, New Haven, Connecticut	06510
(Address of principal executive offices)	(Zip Code)

(203) 404-0410

(Registrant's telephone number, including area code)

Securities registered pursuant to Section 12(b) of the Act:

Title of each class	Trading Symbol	Name of each exchange on which registered
Common Shares, without par value	BHVN	New York Stock Exchange

Securities registered pursuant to Section 12(g) of the Act: **None**

Indicate by check mark if the registrant is a well-known seasoned issuer, as defined in Rule 405 of the Securities Act. Yes ý No o

Indicate by check mark if the registrant is not required to file reports pursuant to Section 13 or Section 15(d) of the Act. Yes o No ý

Indicate by check mark whether the registrant: (1) has filed all reports required to be filed by Section 13 or 15(d) of the Securities Exchange Act of 1934 during the preceding 12 months (or for such shorter period that the registrant was required to file such reports), and (2) has been subject to such filing requirements for the past 90 days. Yes ý No o

Indicate by check mark whether the registrant has submitted electronically every Interactive Data File required to be submitted pursuant to Rule 405 of Regulation S-T (§ 232.405 of this chapter) during the preceding

term loan of $275.0 million that the Borrowers drew at closing in August 2020 (the "Initial Term Loan"), $125.0 million drawn in August 2021 (the "DDTL-2"), and $125.0 million (the "2021 Term Loan") and $100.0 million (the "DDTL-1") both drawn in September 2021. The remaining $125.0 million delayed draw term loan commitments (the "2021 DDTL Commitment") was available to be drawn by the Borrowers until December 31, 2021 (the "Delayed Draw Term Loan Commitment Termination Date"). In November 2021, the Company entered into Amendment No. 3 and Limited Consent to Financing Agreement ("the Third Amendment and Limited Consent") to our Sixth Street Financing Agreement. Pursuant to the Third Amendment and Limited Consent, the lenders consented to the Company's entry into the Collaboration Agreement with Pfizer. In December 2021, we entered into Amendment No. 4 (the "Fourth Amendment") to the Sixth Street Financing Agreement, which extended the Delayed Draw Term Loan Commitment Termination Date to June 30, 2022. For additional details please refer to "Liquidity and Capital Resources" and Note 14, "Debt."

Artizan Biosciences Inc.

In December 2020, we entered into a Series A-2 Preferred Stock Purchase Agreement with Artizan Biosciences Inc. ("Artizan") (Note 14). Under the agreement, we paid Artizan 61,494 shares valued at $6.0 million, which were issued in January 2021. In exchange, we acquired 34,472,031 shares of series A-2 preferred stock of Artizan.

Yale MoDE Agreement

On January 1, 2021, we entered into a worldwide, exclusive license agreement for the development and commercialization of a novel Molecular Degrader of Extracellular Protein (MoDEs) platform based on ground-breaking research conducted in the laboratory of Professor David Spiegel at Yale University (Note 14). Under the agreement, we paid Yale University an upfront cash payment of $1.0 million and 11,668 shares valued at $1.0 million, both of which were included in research and development expense in the consolidated statements of operations.

Consulting Agreement with Moda Pharmaceuticals LLC

On January 1, 2021, we entered into a consulting services agreement with Moda Pharmaceuticals LLC to further the scientific and commercial advancement of technology, drug discovery platforms, product candidates and related intellectual property owned or controlled by us (Note 14). Under the agreement, we paid Moda an upfront cash payment of $2.7 million and 37,836 shares valued at $3.2 million, both of which were included in research and development expense in the consolidated statements of operations and comprehensive loss.

KU Leuven Agreement

In January 2022, Biohaven and Katholieke Universiteit Leuven ("KU Leuven") entered into an Exclusive License and Research Collaboration Agreement (the "KU Leuven Agreement") to develop and commercialize first-in-class TRPM3 antagonists to address the growing proportion of people worldwide living with chronic pain disorders. The TRPM3 antagonist platform was discovered at the Centre for Drug Design and Discovery ("CD3") and the Laboratory of Ion Channel Research ("LICR") at KU Leuven. Under the KU Leuven Agreement, Biohaven receives exclusive global rights to develop, manufacture and commercialize KU Leuven's portfolio of small-molecule TRPM3 antagonists. The portfolio includes the lead candidate, henceforth known as BHV-2100, which has demonstrated promising efficacy in preclinical pain models and will be the first to advance towards Phase 1 studies. Biohaven will support further basic and translational research at KU Leuven on the role of TRPM3 in pain and other disorders. As consideration, KU Leuven received an an upfront cash payment of $3.0 million and 15,340 shares valued at $1.8 million, and is eligible to receive additional development, regulatory, and commercialization milestones payments of up to $327.8 million. In addition, KU Leuven will be eligible to receive mid-single digit royalties on net sales of products resulting from the collaboration.

Kv7 Platform Acquisition

In February 2022, we announced that we entered into a definitive agreement with Channel Biosciences, LLC, a subsidiary of Knopp Biosciences, LLC, to acquire a Kv7 channel targeting platform, adding the latest advances in ion-channel modulation to our growing neuroscience portfolio. BHV-7000 (formerly known as KB-3061) is the lead asset from the Kv7 platform and is a potentially best-in-class potassium channel activator with a profile suggestive of a wide therapeutic index, high selectivity, and significantly reduced GABA-ergic activity. We intend to bring BHV-7000 to the clinic in in 2022 in preparation for a development program in focal epilepsy. In consideration for the transaction, we will make an upfront payment comprised of $65 million in Biohaven common shares and $35 million in cash to Knopp Biosciences. We have also agreed to make additional success-based earnout payments (i) up to $325 million based on BHV-7000 developmental and regulatory epilepsy milestones through approvals in the US, EU and Japan, (ii) up to an additional $250 million based on developmental and regulatory milestones for the Kv7 pipeline development in other indications and additional country approvals, and (iii) up to $562.5 million for commercial sales-based milestones of BHV-7000. Biohaven has also agreed to make scaled royalty payments for BHV-7000 and the pipeline programs, starting at high single digits and peaking at low teens for BHV-7000 and starting at mid-single digits and peaking at low double-digits for the pipeline programs.

Table of Contents

Taldefgrobep Alfa Platform License

In February 2022, following the transfer of intellectual property we announced that we entered into a worldwide license agreement with BMS for the development and commercialization rights to taldefgrobep alfa, a novel, Phase 3-ready anti-myostatin adnectin. The in-licensing of taldefgrobep expands our portfolio of innovative, late-stage product candidates for the treatment of neurologic, neuroinflammatory, and psychiatric indications. Under the terms of the agreement, we will receive worldwide rights to taldefgrobep alfa and BMS will be eligible for regulatory approval milestone payments, as well as tiered, sales-based royalty percentages from the high teens to the low twenties (Note 14). We plan to initiate a Phase 3 clinical trial of taldefgrobep alfa in SMA in 2022.

COVID-19 Update

A novel strain of coronavirus (COVID-19) was first identified in Wuhan, China in December 2019, and subsequently declared a pandemic by the World Health Organization. To date, COVID-19 has surfaced in nearly all regions around the world and resulted in travel restrictions and business slowdowns or shutdowns in affected areas.

Although, as of the date of this Annual Report on Form 10-K, we do not expect any material impact on our long-term activity, the extent to which COVID-19 impacts our business will depend on future developments, which are highly uncertain and cannot be our business, financial condition and results of operations.

The continued spread of COVID-19 could also adversely impact our clinical trial operations. For example, we may be unable to enroll or retain an adequate number of patients to commence or complete our clinical trials, data may be missing, the FDA may delay or terminate clinical trials for any of our product candidates, or primary outcome measures may be impacted. As a result, our ability to generate product revenue from sales of any of those product candidates may be delayed or not realized at all.

Business interruptions from the current or future pandemics may also adversely impact the third parties we rely on to sufficiently manufacture NURTEC ODT and to produce our product candidates in quantities we require, which may impair the commercialization of NURTEC ODT and our research and development activities.

The COVID-19 pandemic and responses to its spread have negatively impacted the global economy, disrupted global supply chains, and created significant volatility and disruption of financial markets. This significant disruption of the global financial markets could reduce our ability to access equity or debt capital on attractive terms if at all, which in turn could negatively affect our liquidity. In addition, a recession or market correction resulting from the spread of COVID-19 could materially affect our business and the value of our common shares.

We have taken numerous steps, and will continue to take further actions, in our

EXHIBIT 39

Secretary of the State of Connecticut
Annual Report

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PRIMARY DETAILS

Business Type:	Foreign
Legal Structure:	LLC
Business Name:	MODA PHARMACEUTICALS LLC
Business ALEI:	US-CT.BER:1322407

	Existing Information	***Updated Information***
Business Email Address:	None	dspiegel256@gmail.com
NAICS Information:	None	Research and Development in Biotechnology (except Nanobiotechnology) (541714)

BUSINESS LOCATION

	Existing Information	***Updated Information***
Principal Office Address:	321 BLAKE CIRCLE HAMDEN, CT 06517	No update
Mailing Address:	321 BLAKE CIRCLE HAMDEN, CT 06517	No update
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Mailing Address (in State of Formation):	1209 ORANGE ST WILMINGTON, DE 19801	No update

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AGENT INFORMATION

Type:	Business
Agent's Name:	C T CORPORATION SYSTEM
Agent's ALEI:	US-CT.BER:0082007

	Existing Addresses	*Updated Addresses*
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CURRENT PRINCIPAL INFORMATION

This section contains principals already on record who remained the same as part of this report; updates to address or title information is displayed as applicable.

Principal Name:	DAVID SPIEGEL	
	Existing Information	***Updated Information***
Title:	MEMBER	No Update
Business Address:	None	No Update
Residence Address:	321 BLAKE CIRCLE, HAMDEN, CT, 06517, United States	No Update

NEW PRINCIPAL INFORMATION

This section contains principals who were added as part of this report.

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Principal Name:	Kathryn Picanso
Title:	Member
Business Address:	None
Residence Address:	321 Blake CirHamden, CT 06517-3351 United States

ACKNOWLEGEMENT

I hereby certify and state under penalties of false statement that all the information set forth on this document is true.

I hereby electronically sign this document on behalf of:

Name of Authorizer: DAVID SPIEGEL
Authorizer Title: MEMBER

Filer Name: David Spiegel
Filer Signature: David Spiegel
Execution Date: 05/21/2022
This signature has been executed electronically

EXHIBIT 40

IN THE UNITED STATES DISTRICT COURT
FOR DISTRICT OF DELAWARE

BIOHAVEN THERAPEUTICS LTD. and YALE UNIVERSITY, Plaintiffs, v. AVILAR THERAPEUTICS, INC., a Delaware corporation, RA CAPITAL MANAGEMENT GP, LLC, a Delaware corporation, and MILIND DESHPANDE, an individual, Defendants.	C.A. No. 1:23-cv-00328-CFC

DEFENDANTS AVILAR THERAPEUTICS, INC. AND RA CAPITAL MANAGEMENT GP, LLC'S FIRST SET OF REQUESTS FOR PRODUCTION OF DOCUMENTS TO PLAINTIFFS BIOHAVEN THERAPEUTICS LTD. AND YALE UNIVERSITY

Pursuant to Rules 26 and 34 of the Federal Rules of Civil Procedure, Defendants Avilar Therapeutics, Inc. and RA Capital Management GP, LLC, by and through their counsel, serve Plaintiffs Biohaven Therapeutics Ltd. and Yale University the following First Set of Requests for Production of Documents ("Requests"). Within thirty (30) days of service, Plaintiffs must produce all documents responsive to the Requests to the office of Wilson, Sonsini, Goodrich & Rosati, P.C., 222 Delaware Avenue, Suite 800, Wilmington, Delaware 19801, or at such other place as may be mutually agreed upon by counsel. Each Request is to be read in accordance with the Definitions and Instructions that follow.

consultants, partners, associates, investigators, representatives, accountants, financial advisors, distributors, and any other person acting on their behalf, pursuant to their authority, or subject to their control.

11. The term "Defendants" means Avilar and RA Capital, and their present and former directors, officers, employees, predecessors in interest, successors in interest, servants, agents, attorneys, consultants, partners, associates, investigators, representatives, accountants, financial advisors, distributors, and any other person acting on their behalf, pursuant to their authority, or subject to their control.

12. The term "Dr. Deshpande" means Defendant Milind Deshpande.

13. The term "Kleo" means Kleo Pharmaceuticals, Inc., and its present and former directors, officers, professors, administrators, employees, predecessors in interest, successors in interest, servants, agents, attorneys, consultants, partners, associates, investigators, representatives, accountants, financial advisors, distributors, and any other person acting on their behalf, pursuant to their authority, or subject to their control.

14. The terms "Moda Entity" and "Moda Entities" mean Moda Pharmaceuticals LLC (Delaware Entity No. 7613172, incorporated Sept. 18, 2019), Moda Pharmaceuticals, Inc. (Delaware Entity No. 7335383, incorporated Mar. 20, 2019), and the Moda Pharmaceuticals LLC referenced in Biohaven's

October 23, 2022 Form 424(b)(4) filing, and their present and former directors, officers, professors, administrators, employees, predecessors in interest, successors in interest, servants, agents, attorneys, consultants, partners, associates, investigators, representatives, accountants, financial advisors, distributors, and any other person acting on their behalf, pursuant to their authority, or subject to their control.

15. The term "Associate" means any employee, contractor, consultant, partner, agent, servant, or any other person hired, contracted, or employed, to act in the interest of an entity, pursuant to delegated authority by an entity, or subject to the control of an entity.

16. The terms "document" and "documents" have the broadest possible meaning under Rule 34(a) of the Federal Rules of Civil Procedure and include "writing(s)" and "recording(s)" under Rule 1001 of the Federal Rules of Evidence, "tangible things" under Rules 26 and 34 of the Federal Rules of Evidence, and all of the following matter in Your actual or constructive possession, custody, or control: all written, typed, printed, recorded, textual, graphic or photographic matter, software, source code, and object code, however produced or reproduced, any notes or drafts, and all copies on which any mark, alteration, writing, or any other change from the original has been made.

DOCUMENT REQUEST 88:

All documents and communications constituting or regarding any agreement, understanding, employment term, policy, or procedure regarding the relationship between the members of the Crews Lab and Yale.

DOCUMENT REQUEST 89:

All documents and communications constituting or regarding any agreement, understanding, employment term, policy, or procedure regarding the relationship between Dr. Spiegel and Kleo, including any documents that are no longer in effect.

DOCUMENT REQUEST 90:

All documents and communications constituting or regarding any agreement, understanding, consultancy, or other arrangement between You and any Moda Entity.

DOCUMENT REQUEST 91:

All documents and communications constituting or regarding any agreement, understanding, consultancy, or other arrangement between Dr. Spiegel and any Moda Entity.

DOCUMENT REQUEST 92:

All documents and communications regarding Biohaven's $5.9 million payment to Moda Pharmaceuticals, LLC.

EXHIBIT 41

JAMA | Original Investigation

Estimated Research and Development Investment Needed to Bring a New Medicine to Market, 2009-2018

Olivier J. Wouters, PhD; Martin McKee, MD, DSc; Jeroen Luyten, PhD

IMPORTANCE The mean cost of developing a new drug has been the subject of debate, with recent estimates ranging from $314 million to $2.8 billion.

OBJECTIVE To estimate the research and development investment required to bring a new therapeutic agent to market, using publicly available data.

DESIGN AND SETTING Data were analyzed on new therapeutic agents approved by the US Food and Drug Administration (FDA) between 2009 and 2018 to estimate the research and development expenditure required to bring a new medicine to market. Data were accessed from the US Securities and Exchange Commission, Drugs@FDA database, and ClinicalTrials.gov, alongside published data on clinical trial success rates.

EXPOSURES Conduct of preclinical and clinical studies of new therapeutic agents.

MAIN OUTCOMES AND MEASURES Median and mean research and development spending on new therapeutic agents approved by the FDA, capitalized at a real cost of capital rate (the required rate of return for an investor) of 10.5% per year, with bootstrapped CIs. All amounts were reported in 2018 US dollars.

RESULTS The FDA approved 355 new drugs and biologics over the study period. Research and development expenditures were available for 63 (18%) products, developed by 47 different companies. After accounting for the costs of failed trials, the median capitalized research and development investment to bring a new drug to market was estimated at $1141.7 million (95% CI, $888.1 million-$1480.8 million), and the mean investment was estimated at $1559.1 million (95% CI, $1271.0 million-$1893.8 million) in the base case analysis. Median estimates by therapeutic area (for areas with ≥5 drugs) ranged from $765.9 million (95% CI, $323.0 million-$1473.5 million) for nervous system agents to $2771.6 million (95% CI, $2051.8 million-$5366.2 million) for antineoplastic and immunomodulating agents. Data were mainly accessible for smaller firms, orphan drugs, products in certain therapeutic areas, first-in-class drugs, therapeutic agents that received accelerated approval, and products approved between 2014 and 2018. Results varied in sensitivity analyses using different estimates of clinical trial success rates, preclinical expenditures, and cost of capital.

CONCLUSIONS AND RELEVANCE This study provides an estimate of research and development costs for new therapeutic agents based on publicly available data. Differences from previous studies may reflect the spectrum of products analyzed, the restricted availability of data in the public domain, and differences in underlying assumptions in the cost calculations.

Editorial pages 826, 829, and 831

Supplemental content

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JAMA. 2020;323(9):844-853. doi:10.1001/jama.2020.1166
Corrected on September 20, 2022.

Rising drug prices have attracted public debate in the United States and abroad on fairness of drug pricing and revenues.[1] Central to this debate is the scale of research and development investment by biopharmaceutical companies that is required to bring new medicines to market.[2]

The most widely cited studies of the cost of developing a new drug (DiMasi et al[3,4]) reported a sharp increase in the mean cost of developing a single new therapeutic agent from $1.1 billion in 2003 to $2.8 billion in 2013 (in 2018 US dollars), based on a real cost of capital rate of 11% per year in the former study[3] and of 10.5% per year in the latter.[4] Other studies in this period, most of which relied on confidential or proprietary data, reported figures from $314 million to $2.1 billion (in 2018 US dollars).[5-11]

In 2017, Prasad and Mailankody estimated the research and development costs of new cancer drugs using public data reported by pharmaceutical firms to the US Securities and Exchange Commission (SEC).[12] They estimated the median research and development cost of bringing a single cancer drug to market to be $780 million (in 2018 US dollars), capitalized at a real cost of capital rate of 7% per year, based on a sample of 10 drugs.[12]

This present study estimates the research and development investment required to bring a new therapeutic agent to market using publicly available data for products approved by the US Food and Drug Administration (FDA) between 2009 and 2018.

Key Points

Question How much do drug companies spend on research and development to bring a new medicine to market?

Findings In this study, which included 63 of 355 new therapeutic drugs and biologic agents approved by the US Food and Drug Administration between 2009 and 2018, the estimated median capitalized research and development cost per product was $1.1 billion, counting expenditures on failed trials. Data were mainly accessible for smaller firms, products in certain therapeutic areas, orphan drugs, first-in-class drugs, therapeutic agents that received accelerated approval, and products approved between 2014 and 2018.

Meaning This study provides an estimate of research and development costs for new therapeutic agents based on publicly available data; differences from previous studies may reflect the spectrum of products analyzed and the restricted availability of data in the public domain.

Methods

Sample Identification and Characteristics

We identified all new therapeutic agents, ie, new drug applications and biologics license applications approved by the FDA between 2009 and 2018, in the Drugs@FDA database.[13] For each, we extracted the date of approval, date of submission of investigational new drug application, date of submission of new drug application or biologics license application, indication, type (pharmacologic or biologic), expedited programs (priority review, accelerated approval, fast track, or breakthrough), orphan status, route of administration (oral, injection, intravenous, or other), and manufacturer (eTable 1 in the Supplement). To capture innovation, we determined whether an agent was first in class using publications by FDA officials.[14,15] We checked the data for consistency with published reports.[15,16]

Therapeutic areas were obtained from the anatomical therapeutic chemical classification system database.[17] For agents that were not yet classified, we based our decision on the approved indication.

For each agent, we identified start and end dates of clinical studies (phases 1, 2, and 3 for the FDA-approved indication) from ClinicalTrials.gov (search conducted on April 4, 2019). If there were multiple studies in the same phase, the earliest start date was selected. We verified these dates with reports in SEC filings and used the dates from SEC filings if there were discrepancies. We classified combined phase 1 and 2 trials as phase 2 and combined phase 2 and 3 trials as phase 3, consistent with other studies.[18-20] Dates of submission of investigational new drug applications were used to approximate the end of preclinical testing; these dates were checked for consistency with filings to ensure clinical testing had not already begun outside the United States.

No data were collected from human participants, and all data in this study were publicly available.

Research and Development Data Extraction

Publicly traded US companies are legally required by the SEC to file annual 10-K and quarterly 10-Q forms, which are reports of key financial performance indicators that include audited financial statements and data on research and development expenditures. For every agent in our sample, we searched the SEC website for reports from the firm that received FDA approval for it.[21]

Exclusions

As reports for private US drug firms and foreign companies listed on non-US stock exchanges were unavailable, their products were excluded. For firms with available reports, we screened 10-K and 10-Q filings for data on research and development expenditures on individual drug candidates. We excluded products developed by companies that only reported total research and development expenditures across all drug candidates or across therapeutic areas.

For excluded products, we searched the 10-K and 10-Q forms and online press releases of manufacturers at the time that agents were approved to see if any were developed in collaboration with other firms via licensing deals. If so, we searched for 10-K and 10-Q forms from those firms in case there were research and development data for the product in question.

Inclusions

For each therapeutic agent with available data, we extracted direct and indirect research and development expenditures in each year of development. Drugs were tracked across years in

SEC filings using the brand, generic, or compound names of agents, as appropriate.

Direct research and development expenses included all resources directly allocated to a particular agent. Indirect research and development expenses, which included personnel and overhead costs, were sometimes reported as a lump sum across all drug development programs. If so, we applied the same percentage of direct research and development costs attributable to a particular agent to estimate indirect costs for the same agent. The proportional allocation of personnel and overhead expenses is common practice in costing studies.[22]

Costs were tracked from the year a company started reporting costs for a particular drug candidate in their financial statements until the quarter of approval, which often included 1 or more years of preclinical costs. In some cases, at the first mention of the candidate in SEC filings, companies reported the costs incurred since inception of the drug development program. Certain companies only started tracking costs at late stages of preclinical development or at the start of phase 1 of development, resulting in an underreporting of preclinical costs.

Some drugs were initially developed by companies that subsequently licensed out their drug candidates to other firms, which then brought these products to market. In these cases, it was assumed that any preclinical and clinical costs incurred during initial development was included in licensing fees and milestone payments. Hence, where these fees and payments were recorded as research and development expenses for the agent in question, these costs were extracted. Data on costs incurred by the originator firms were not collected.

If SEC filings were missing for 3 or fewer years since the inception of the drug development program (eg, if a company was privately held during early years of development) and the product did not move between development phases (ie, either from 1 to 2 or 2 to 3), we extrapolated costs from the closest available year. Products were excluded if more than 3 years of SEC filings were missing.

Three investigators independently extracted all research and development data used in this study. Discrepancies were resolved through discussions. Where disagreements existed, we assumed the higher estimate of research and development expenditures.

Quality Assessments

Consistency and completeness of company reporting in SEC filings varied over time. Many reported detailed research and development costs, which allowed us to track outlays over time for individual candidates. Others reported costs inconsistently or with missing data for some years, requiring various assumptions, for example on timings of transitions between phases and extrapolations when SEC filings were missing.

To aid interpretation, we categorized each estimate as high, medium, or low quality, depending on the availability and consistency of reported data. The categorization was developed through discussion between all authors.

High-quality estimates comprised drugs discovered internally, allowing tracking of costs back to inception of the development program, and products licensed at preclinical or phase 1 stages with minimal up-front fees or milestone payments captured in SEC filings. Late commercialization deals related to marketing of products in non-US markets were also deemed high-quality estimates, as they would have had little or no effect on research and development expenses incurred on trials required for FDA approval.

Low-quality estimates comprised all acquisitions, licensing deals, or other collaboration agreements in phases 2 or 3, earlier deals in which it was unclear whether all costs were captured in data extraction, and estimates requiring extrapolation of 2 to 3 years of data. We classified estimates as medium quality when other judgment calls regarding financial reporting, as agreed upon by the authors, had to be made.

Two investigators independently categorized the quality of estimates and resolved discrepancies through discussions.

Costs of Failed Trials

Accurate information on costs of failures, ie, research and development outlays on candidates being developed by companies but not ultimately approved, is essential to estimating the costs of drug development. We accounted for failures using data on aggregate clinical trial success rates from a recent study by Wong et al (**Table 1**).[18]

Wong et al reported that the percentages of FDA approvals were 13.8% for therapeutic agents entering phase 1, 21.0% for those entering phase 2, and 59.0% for those entering phase 3.[18]

Wong et al[18] provided success rates through phase 3. We supplemented these rates with a recent estimate of the proportion of biologics license applications and new drug applications that are approved by the FDA (83.2%).[20]

Costing Method

For each agent, we estimated the expected research and development investment to bring the drug to market in 3 steps.

First, we summed direct and indirect research and development spending on a therapeutic agent in each year. All sums were inflation adjusted to 2018 dollars using the US consumer price index.

Second, we accounted for failed projects by dividing total research and development expenditures on a drug in a particular year by the corresponding aggregate phase-specific probability of success, similar to what was done in previous studies of costs of drug development.[3-7] For example, for each drug, we divided phase 1 costs in each year by 0.138, which accounted for spending on the other 6.2 phase 1 trials that would fail, on average, for each successful development program. We used phase 1 rates to adjust preclinical expenditures, and we used the proportion of biologics license applications and new drug applications that are approved by the FDA to adjust costs once these applications were submitted to the agency for regulatory approval. Licensing fees and milestone payments, where captured, were adjusted using the success rate for the trial phase that was ongoing when the payments were made. When a phase shift took place within the financial year, we allocated the cost proportionally to the time spent in each phase. For example, if development moved from phase 1 to phase 2 on July 1 of a given year, we divided the costs equally be-

Table 1. Clinical Trial Success Rates by Phase (on Aggregate and by Therapeutic Area)[a]

Source	Phase 1 to Approval, %[b]	Phase 2 to Approval, %[c]	Phase 3 to Approval, %[d]	FDA Submission to Approval, %[e]
Aggregate rates				
Wong et al[18]	13.8	21.0	59.0	83.2
Thomas et al[19]	9.6	15.3	49.6	85.3
Hay et al[20]	10.4	16.2	50.0	83.2
Therapeutic-area-specific rates[18]				
Oncology	3.4	6.7	35.5	81.7
Metabolism and endocrinology	19.6	24.1	51.6	80.4
Cardiovascular	25.5	32.3	62.2	84.5
Central nervous system	15.0	19.5	51.1	82.2
Autoimmune and inflammation	15.1	21.2	63.7	80.3
Ophthalmology[f]	32.6	33.6	74.9	80.4
Infectious disease	25.2	35.1	75.3	84.9
Other[g]	20.9	27.3	63.6	80.4

Abbreviation: FDA, US Food and Drug Administration.

[a] Rates across all indications for individual therapeutic agents (as opposed to rates for lead indications, which were higher in all phases). Only the success rates used in this analysis were reported.

[b] Phase 1 trials, which usually include as many as 100 healthy volunteers and may take several months to conduct, are primarily used to assess the tolerability and safety of a therapeutic agent in different doses; these are sometimes referred to as first-in-human trials.

[c] Phase 2 trials, which can involve as many as a few hundred patients with a disease or condition and take several months to 2 years to complete, are typically used to gather data on the efficacy and safety of a therapeutic agent in different doses.

[d] Phase 3 trials, which can involve several thousand participants with a disease or condition and may take 1 to 4 years to run, are generally used to confirm the efficacy and safety of the dose of the therapeutic agent believed to provide the best risk-benefit ratio.[23]

[e] Indicates the proportion of new drug applications and biologics license applications approved by the FDA. Wong et al[18] reported aggregate and therapeutic-area–specific rates through phase 3. These data were supplemented with estimates of FDA submission to approval rates from Hay et al; if a particular category from the study by Wong et al was not reported by Hay et al, the category *Other* was used.[20]

[f] This category was applied to therapeutic agents classified as treating sensory organ diseases, ie, anatomical therapeutic chemical classification system code *S*.

[g] Values in this category were based on the rates for "all [agents] without oncology" reported by Wong et al.[18] These rates were applied to therapeutic agents that were outside the other categories.

tween each phase. Similarly, in the year of approval, we multiplied the total cost by the fraction of the year elapsed by the time of approval. Hence, if a drug was approved on July 1, we only counted 50% of the costs in the year of approval since firms often incurred postapproval costs related to pharmacovigilance or testing in other indications.

Third, we applied a real cost of capital rate of 10.5% per year (ie, weighted average cost of capital in the pharmaceutical industry), as in the DiMasi et al study.[4] Cost of capital is the required rate of return for an investor and encapsulates a risk-free rate (ie, opportunity cost) and premium based on the likelihood of business failure.[24]

Sensitivity and Subgroup Analyses

We ran 4 univariate sensitivity analyses. First, as the results were sensitive to the choice of aggregate clinical trial success rates (by phase), we recalculated the results using aggregate rates reported in 2 other studies (Table 1).[19,20] Next, we calculated a second estimate of research and development costs using therapeutic-area–specific rates reported by Wong et al (Table 1), instead of aggregate rates. For example, oncology drugs in phase 1 have a 3.4% chance of ultimately receiving FDA approval, so we divided each year of phase 1 costs for these products by 0.034. Third, we performed a rerun of the analyses using a real cost of capital rate of 7% (as done by Prasad and Mailankody[12]) and 0% (to show noncapitalized outlays). Fourth, to account for potentially missing preclinical expenditures, we adopted the same assumption around preclinical costs as DiMasi et al, who reported that preclinical costs represented 42.9% of their total research and development estimate.[4] Thus, for each product in our sample, we isolated clinical expenditures and imputed a preclinical cost that amounted to this percentage. No imputations were performed for products acquired through purchase after clinical development had begun since it was assumed that licensing fees and milestone payments reflected preclinical costs incurred by the company that sold the rights to the product. Additionally, we ran another sensitivity analysis but with imputations done for all products, including agents acquired through purchase.

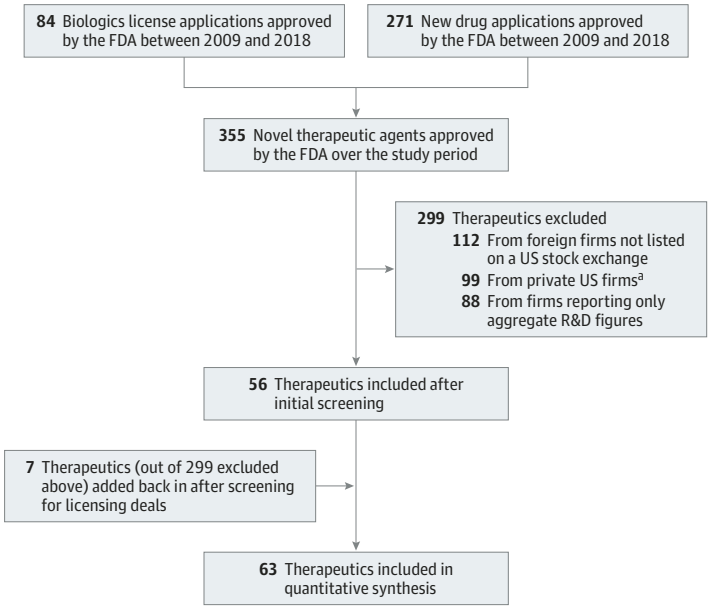
As a subgroup analysis, we reported mean and median amounts by therapeutic area, using area-specific rates to adjust for costs of failure.

Statistical Analysis

We estimated the mean and median research and development investments across our sample in the base case and sensitivity analyses. We then restricted the sample to high-quality estimates and recalculated the mean and median amounts.

We conducted a nonparametric bootstrapped resampling with replacement (1000 iterations) to calculate 95% CIs around the estimated mean and median investments in research and development in our sample. We used χ^2 tests to identify statistically significant differences in characteristics of the study sample vs therapeutic agents approved by the FDA between

Figure 1. Selection Process for Therapeutic Agents



FDA indicates the US Food and Drug Administration; R&D, research and development.

[a] Includes firms that went public while developing the therapeutic agent in question but for which US Securities and Exchange Commission (SEC) filings were missing for more than 3 years of the drug development period.

2009 and 2018 that were excluded from our analysis. We used Kruskal-Wallis and Mann-Whitney *U* tests, as appropriate, to identify statistically significant differences in median estimated research and development investments across therapeutic areas and other drug characteristics.

All statistical tests were 2-tailed and used a type I error rate of 0.05. The data were analyzed using Stata version 15 (StataCorp).

Results

Between 2009 and 2018, the FDA approved 355 new drugs and biologics. Research and development expenditures from SEC government filings were available for 63 of these products, developed by 47 different companies (**Figure 1**). The sample covered 17.7% (63/355) of all new therapeutic agents approved by the FDA over this 10-year period. Twenty-three of the estimates were judged of high quality, 18 medium quality, and 22 low quality. eTable 2 in the Supplement provides the rationale for the quality categorization of each agent.

Sample Characteristics

Table 2 presents statistics for the 63 included therapeutic agents. The sample contained a larger proportion of orphan drugs, therapeutic agents that benefited from expedited development or approval pathways, and first-in-class drugs compared with all FDA-approved products between 2009 and 2018, although these differences were not statistically significant. Differences in the breakdown of products by therapeutic area, accelerated vs regular approval, and approval dates were statistically significant.

Research and Development Investments

Without adjustments for costs of failed trials, no statistically significant differences in the median research and development investment required to bring a new drug to market were observed across any of the drug characteristics shown in Table 2, except median costs for biologic drugs that were higher than those for pharmacologic drugs (eTable 3 in the Supplement). For the 63 agents included in the analysis, outlays for research and development (ie, total noncapitalized direct and indirect expenses incurred during preclinical and clinical testing) were estimated at a median of $319.3 million (95% CI, $236.4 million-$351.4 million), and the estimated mean outlay was $374.1 million (95 CI, $301.9 million-$464.2 million), (eTable 4 in the Supplement). The mean (SD) number of years of data per drug was 8.3 (2.8) years. eTable 5 in the Supplement shows the dates of phase changes for clinical trials of each included agent.

After accounting for costs of failed trials, the estimated median research and development investment required to bring a new drug to market, capitalized at a rate of 10.5% per year, was $1141.7 million (95% CI, $888.1 million-$1480.8 million), and the estimated mean was $1559.1 million (95% CI, $1271.0 million-$1893.8 million) (**Table 3**). **Figure 2** shows point estimates for each of the 63 agents, which ranged from $143.2 million for Mytesi (crofelemer) to $7424.2 million for Dupixent (dupilumab).

Restricting the analysis to high-quality estimates (n = 23), the estimated median research and development investment increased from $1141.7 million to $1310.5 million (95% CI, $963.0 million-$1392.2 million), while the estimated mean declined from $1559.1 million to $1383.8 million (95% CI, $1077.7 million-$1751.3 million).

Table 2. Characteristics of New Therapeutic Agents Approved by the US Food and Drug Administration Between 2009 and 2018[a]

Characteristics	No. (%) Included Agents (n = 63)	No. (%) Full Sample (n = 355)	*P* Value
Agent type			
Pharmacologic	47 (75)	271 (76)	.72
Biologic	16 (25)	84 (24)	
Therapeutic area[b]			
Antineoplastic and immunomodulating agents	20 (32)	116 (33)	.02
Alimentary tract and metabolism	15 (24)	44 (12)	
Nervous system	8 (13)	33 (9)	
Antiinfective agents for systemic use	5 (8)	40 (11)	
Other	15 (24)	122 (34)	
Orphan drug	31 (49)	145 (41)	.14
Drug received accelerated approval	14 (22)	41 (12)	.003
Drug benefited from any expedited development or approval pathway[c]	48 (76)	234 (66)	.06
Innovativeness			
First in class	27 (43)	127 (36)	.20
Next in class	36 (57)	228 (64)	
Route of administration[d]			
Oral	28 (44)	187 (53)	.20
Injection	20 (32)	87 (25)	
Intravenous	10 (16)	41 (12)	
Other	5 (8)	40 (11)	
Approval dates			
2009-2013	17 (27)	142 (40)	.02
2014-2018	46 (73)	213 (60)	

[a] Analyses were carried out using χ^2 tests comparing the data for included agents (n = 63) vs excluded ones (n = 292).

[b] Other therapeutic areas included blood and blood-forming organs, cardiovascular system, dermatologicals, musculoskeletal system, sensory organs, and various.

[c] Pathways included accelerated approval, breakthrough therapy, fast track, orphan drug, and priority review.

[d] Injection included intramuscular and subcutaneous; other routes included multiple, ophthalmic, and topical.

Sensitivity Analyses

Table 3 shows the results of univariate sensitivity analyses. When the aggregate success rates reported by Hay et al[20] were used instead of those reported by Wong et al,[18] the estimated median research and development investment, capitalized at a rate of 10.5% per year, increased from $1141.7 million to $1404.9 million (95% CI, $1102.2 million-$1773.4 million), while the estimated mean increased from $1559.1 million to $1976.6 million (95% CI, $1595.5 million-$2454.8 million). When the rates from Thomas et al[19] were used, the estimated median research and development investment, capitalized at an annual rate of 10.5%, was $1465.8 million (95% CI, $1121.5 million-$1887.1 million), and the estimated mean was $2059.5 million (95% CI, $1639.9 million-$2511.7 million).

When therapeutic-area-specific rates from Wong et al,[18] rather than aggregate rates, were used to account for costs of failed trials for each agent, the estimated median research and development investment, capitalized at a rate of 10.5% per year, increased from $1141.7 million to $1385.2 million (95% CI, $1053.9 million-$1971.8 million), and the estimated mean rose from $1559.1 million to $2307.2 million (95% CI, $1726.9 million-$3013.0 million).

When the costs were capitalized at an annual rate of 7% instead of 10.5%, the median expected investment decreased from $1141.7 million to $998.2 million (95% CI, $801.0 million-$1317.8 million), and the mean decreased from $1559.1 million to $1352.4 million (95% CI, $1085.9 million-$1653.7 million). When costs were not capitalized, rather than capitalized at an annual rate of 10.5%, the median expected investment decreased from $1141.7 million to $770.2 million (95% CI, $645.6 million-$939.1 million), and the mean decreased from $1559.1 million to $1032.0 million (95% CI, $845.2 million-$1243.3 million).

With the adjustments for potentially missing preclinical costs done for 33 of 63 products (ie, excluding agents acquired through purchase), based on the DiMasi et al[4] approach, the estimated median research and development investment increased from $1141.7 million to $1620.5 million (95% CI, $1139.8 million-$2255.1 million), while the estimated mean increased from $1559.1 million to $2123.1 million (95% CI, $1671.2 million-$2670.3 million). With the adjustments for potentially missing preclinical costs done for all 63 products, the estimated median research and development investment increased to $1787.2 million (95% CI, $1514.5 million-$2438.2 million), while the estimated mean increased to $2600.6 million (95% CI, $2071.7 million-$3200.7 million).

Restricting the sensitivity analyses to high-quality estimates (n = 23), the estimated median and mean research and development investments required to bring a new drug to market increased in most cases (Table 3). eTable 6 in the Supplement shows the estimates for each agent in the base case and sensitivity analyses.

Table 3. Median Expected Research and Development Expenditure on New Therapeutic Agents Approved by the US Food and Drug Administration (2009-2018) in Main and Sensitivity Analyses

Parameter Varied in Sensitivity Analysis	Research and Development Costs in US$, Millions (95% CI)	
	All Included Agents (n = 63)	High-Quality Sample (n = 23)
Source of aggregate clinical trial success rates		
Wong et al (base case)[18]	1141.7 (888.1-1480.8)	1310.5 (963.0-1392.2)
Hay et al[20]	1404.9 (1102.2-1773.4)	1620.3 (1191.8-1773.4)
Thomas et al[19]	1465.8 (1121.5-1887.1)	1678.4 (1259.7-1999.3)
Aggregate vs therapeutic-area-specific success rates		
Aggregate rates (base case)[18]	1141.7 (888.1-1480.8)	1310.5 (963.0-1392.2)
Area-specific rates[18]	1385.2 (1053.9-1971.8)	1220.1 (994.3-2118.3)
Cost of capital rate		
10.5% (base case)	1141.7 (888.1-1480.8)	1310.5 (963.0-1392.2)
7%	998.2 (801.0-1317.8)	1075.0 (851.1-1317.8)
0%	770.2 (645.6-939.1)	832.8 (669.6-1040.9)
Adjustment for potential underreporting of spending on preclinical trials		
No (base case)	1141.7 (888.1-1480.8)	1310.5 (963.0-1392.2)
Yes, excluding agents acquired through purchase	1620.5 (1139.8-2255.1)	1717.7 (1453.6-2255.1)
Yes, including all products	1787.2 (1514.5-2438.2)	2194.1 (1620.5-2404.0)

Subgroup Analyses by Therapeutic Area

Median estimates by therapeutic area (for areas with ≥5 drugs), adjusted using area-specific rates and capitalized at 10.5% per year, ranged from $765.9 million (95% CI, $323.0 million-$1473.5 million) for nervous system agents to $2771.6 million (95% CI, $2051.8 million-$5366.2 million) for antineoplastic and immunomodulating agents. The corresponding mean estimates ranged from $1076.9 million (95% CI, $508.7 million-$1847.1 million) for nervous system agents to $4461.2 million (95% CI, $3114.0 million-$6001.3 million) for antineoplastic and immunomodulating agents (**Table 4**).

Discussion

Based on data for 63 therapeutic agents developed by 47 companies between 2009 and 2018, the median research and development investment required to bring a new drug to market was estimated to be $1142 million, and the mean was estimated to be $1559 million. Estimates differed across therapeutic areas, with costs of developing cancer drugs the highest. The results included costs of failed clinical trials and varied in sensitivity analyses using different estimates of trial success, preclinical expenditures, and cost of capital.

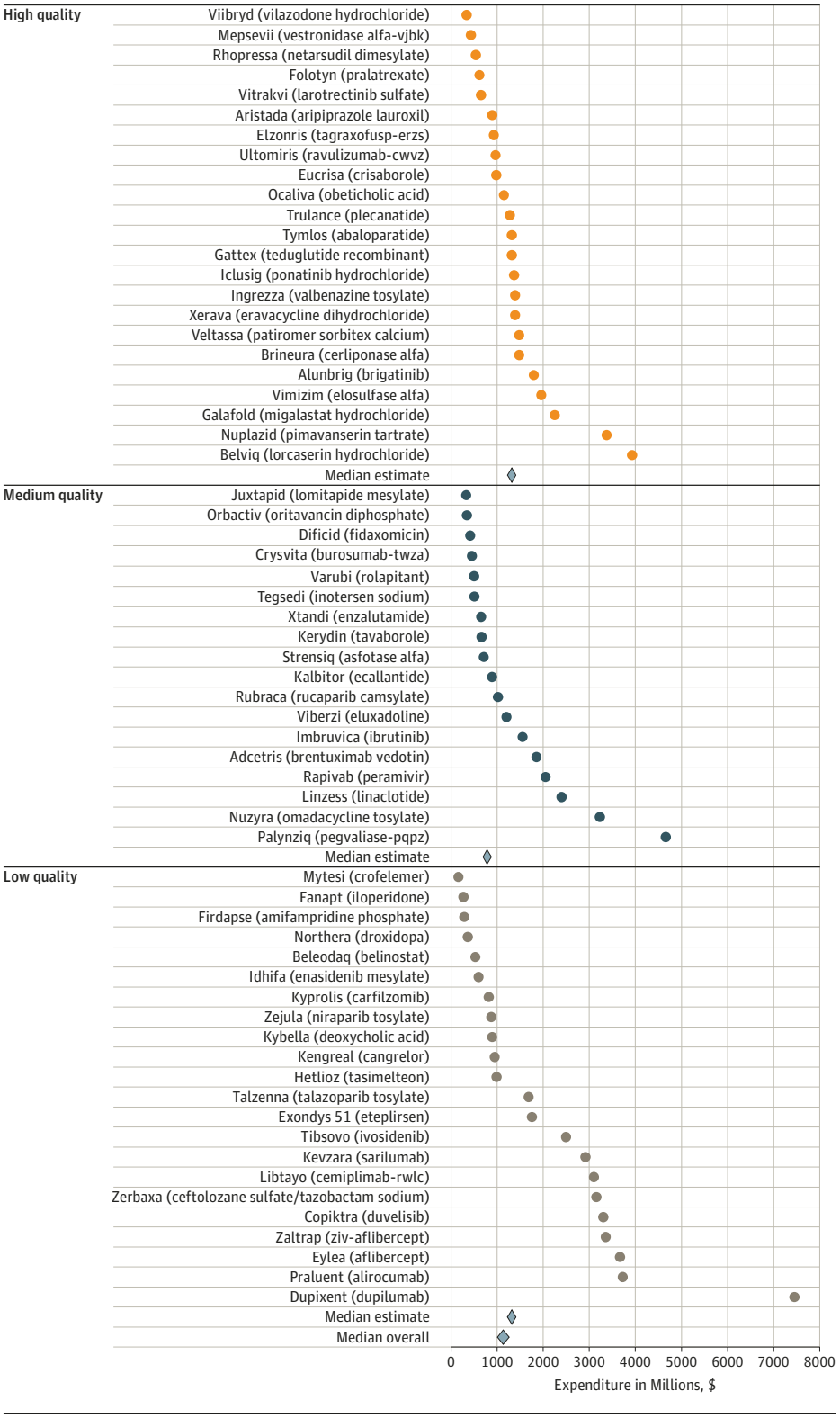
These figures were higher than the median capitalized research and development cost of $780 million (in 2018 US dollars) reported by Prasad and Mailankody for oncology drugs.[12] This may be because adjustments based on clinical trial success rates were applied in the present study to account for costs of failures, whereas Prasad and Mailankody restricted their analysis to companies bringing their first drug to market and then summed the total research and development expenditures of each company during the development periods of the drugs in their sample. Most of the companies included in their study appeared to be more successful than the average company.[25-27] Moreover, their analysis was based on data for 10 oncology drugs, which limits the comparability of their results with the present study.

The mean estimate of $1.6 billion in the present study was lower than the $2.8 billion (in 2018 US dollars) reported by DiMasi et al, which was based on data for 106 products developed by 10 large firms.[4] The estimate by DiMasi et al used confidential data on costs voluntarily submitted by anonymous companies without independent verification, making them difficult to validate.[12,28-30] The higher estimate of DiMasi et al seems to reflect a combination of higher clinical costs incurred by larger drug developers, lower estimates of trial success for each stage of development compared to the more recent data presented by Wong et al, and different assumptions about preclinical expenditures as their data set did not permit allocating these expenditures to specific agents.

The results of the present study varied widely when subject to sensitivity analyses, especially using different success rates. The methods employed by Wong et al to handle missing data were an improvement on earlier studies of trial success rates, and their study was based on a larger sample.[18] Wong et al also noted that the most cited studies of success rates[19,20,31] originated from researchers with ties to the pharmaceutical industry, and elaborated that "previous estimates of drug development success rates [relied] on relatively small samples from databases curated by the pharmaceutical industry and [were] subject to potential selection biases."[18] Also, compared with these earlier studies of success rates,[19,20,31] the timing of the work by Wong et al[18] more closely aligned with that of the present study, thereby improving its internal validity.

There are challenges in isolating preclinical investments by drug companies. It is especially difficult to identify the exact date from which costs should start being allocated to individual agents during the early stages of preclinical research. The base case scenario in this study relied on preclinical costs reported by firms in SEC filings, which were likely underestimated since many companies did not attribute costs during the drug discovery stages to individual candidates. DiMasi et al estimated that preclinical costs accounted, on average, for 42.9% of total capitalized costs, based on aggregated data on preclinical spending and assumptions around the duration of preclinical testing.[4] Although preclinical costs were variously estimated in the present study, including indirectly through license fees, preclinical data were directly captured for 19 products. For these products, preclinical costs generally accounted for a lower share of the total capitalized costs (ranging from 0.3% to 40.5%; median 9.6%) than what was estimated by DiMasi et al (eTable 4 in the Supplement). For comparison, however, the 42.9% estimate was used to impute pre-

Figure 2. Estimated Expenditures for Therapeutic Agents by Quality of the Estimates



Indicates the estimated research and development investment needed for an average company to bring one of these products to market based on aggregate clinical trial success rates. Individual companies may have spent more or less on their respective development pipelines, based on their individual success rates.

clinical costs in sensitivity analyses in this study. Further validation work is needed to establish the preclinical share of research and development estimates for individual products.

Greater transparency around research and development costs is essential for analysts to check the veracity of claims by companies that the steep prices of new drugs are driven by

Table 4. Mean And Median Expected Research and Development Expenditure on New Therapeutic Agents Approved by the US Food and Drug Administration (2009-2018) by Therapeutic Area

Therapeutic Area[a]	Sample Size	Expenditure in US$, Millions (95% CI)[b]	
		Median	Mean
Antineoplastic and immunomodulating agents	20	2771.6 (2051.8-5366.2)	4461.2 (3114.0-6001.3)
Alimentary tract and metabolism	15	1217.6 (613.9-1792.4)	1430.3 (920.8-2078.7)
Nervous system	8	765.9 (323.0-1473.5)	1076.9 (508.7-1847.1)
Antiinfectives for systemic use	5	1259.9 (265.9-2128.3)	1297.2 (672.5-1858.5)
Dermatologicals	4	747.4	1998.3
Cardiovascular system	3	339.4	1152.4
Musculoskeletal system	3	1052.6	937.3
Blood and blood-forming organs	2	793.0	793.0
Sensory organs	2	1302.8	1302.8
Other[c]	1	1121.0	1121.0

[a] Therapeutic areas were obtained from the anatomical therapeutic chemical classification system database.[17] Where agents were not yet classified, the categorization was based on the approved indication.

[b] Bootstrapped CIs were not calculated for therapeutic areas with less than 5 samples. Estimates were based on therapeutic-area–specific success rates reported by Wong et al.[18]

[c] The product Veltassa (patiromer sorbitex calcium) was assigned to the therapeutic area *Various* under the subgroup *Drugs for treatment of hyperkalemia and hyperphosphatemia*.

high development outlays. While these expenditures are undoubtedly high, as shown in this study, it is important for policy makers, regulators, and payers to know the exact scale of these investments. This knowledge can inform the design of pricing policies that give adequate rewards for innovative drugs that bring value to health care systems.

Limitations

This study has several limitations. First, data were unavailable for many products approved by the FDA during the study period. No data were available for products developed by non-US companies not listed on a US stock exchange and large drug firms that did not report research and development figures for individual drug candidates. Thus, there was likely an overrepresentation of smaller firms, which may have run leaner operations than larger ones. This limited the generalizability of the results to all products.

Second, the included agents differed from other drugs approved by the FDA between 2009 and 2018, although not all differences were statistically significant. The sample included a larger proportion of orphan drugs, products in certain therapeutic areas, first-in-class drugs, therapeutic agents that received accelerated approval, and products approved between 2014 and 2018.

Third, there were inconsistencies in research and development reporting between companies, which made it difficult to ensure perfect comparability of research and development figures between firms. These inconsistencies may have been explained by differences in accounting policies. For instance, some firms allocated overhead and administrative costs to direct research and development figures, while others reported these costs separately. Some reported preclinical research costs as a separate line item, while others incorporated them in overhead costs. Companies also reported costs associated with licensing deals, drug acquisitions, and collaboration agreements differently, so it is likely that not all costs were fully reflected in some estimates.

Fourth, uncertainties in the analysis may have resulted in under- or overestimations of research and development expenditures for some products. It is difficult to attribute costs to individual drug candidates in the early stages of preclinical development, so only the costs reported by firms in SEC filings were considered in the base case analysis. However, since preclinical costs may have been underreported by some companies, sensitivity analyses were conducted to produce an upper-bound estimate of preclinical expenditures. Conversely, many drug firms conducted trials for a particular candidate for multiple indications, which may have led to overestimations of costs since trial expenditures were not broken down by indication but instead reported as annual lump sums for each agent. Also, the estimates did not reflect any public tax credits or subsidies, which may have led to further overestimations of costs incurred by companies.

Conclusions

This study provides an estimate of research and development costs for new therapeutic agents based on publicly available data. Differences from previous studies may reflect the spectrum of products analyzed, the restricted availability of data in the public domain, and differences in underlying assumptions in the cost calculations.

ARTICLE INFORMATION

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Correction: This article was corrected on September 20, 2022, to fix multiple data points affected by a correction in one of the sources used in some of the original calculations; affected data were corrected in the abstract, text, several tables, Figure 2, and the Supplement.

Author Contributions: Dr Wouters had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Concept and design: Wouters.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Wouters.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Wouters.
Administrative, technical, or material support: All authors.
Supervision: Wouters.

Conflict of Interest Disclosures: Dr McKee reported receipt of grants from the Wellcome Trust, European Commission, and United Kingdom Research and Innovation outside the submitted work. No other disclosures were reported.

Data Sharing Statement: All data used in this study were in the public domain. An example data extraction file is available from the corresponding author upon request.

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EXHIBIT 42

LYTACs That Engage a Liver-Specific ASGPR for Targeted Protein Degradation

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The most recent Bertozzi manuscript:

https://chemrxiv.org/articles/preprint/Lysosome_Targeting_Chimeras_LYTACs_That_Engage_a_Liver-Specific_Asialoglycoprotein_Receptor_for_Targeted_Protein_Degradation/12736778

EXHIBIT 43

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Determination of the Upper Size Limit for Uptake and Processing of Ligands by the Asialoglycoprotein Receptor on Hepatocytes *in Vitro* and *in Vivo**

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The asialoglycoprotein receptor (ASGPr) on hepatocytes plays a role in the clearance of desialylated proteins from the serum. Although its sugar preference (*N*-acetylgalactosamine (GalNAc) ≫ galactose) and the effects of ligand valency (tetraantennary > triantennary ≫ diantennary ≫ monoantennary) and sugar spacing (20 Å ≫ 10 Å ≫ 4 Å) are well documented, the effect of particle size on recognition and uptake of ligands by the receptor is poorly defined. In the present study, we assessed the maximum ligand size that still allows effective processing by the ASGPr of mouse hepatocytes *in vivo* and *in vitro*. Hereto, we synthesized a novel glycolipid, which possesses a highly hydrophobic steroid moiety for stable incorporation into liposomes, and a triantennary $GalNAc_3$-terminated cluster glycoside with a high nanomolar affinity (2 nM) for the ASGPr. Incorporation of the glycolipid into small (30 nm) [^{3}H]cholesteryl oleate-labeled long circulating liposomes (1–50%, w/w) caused a concentration-dependent increase in particle clearance that was liver-specific (reaching 85 ± 7% of the injected dose at 30 min after injection) and mediated by the ASGPr on hepatocytes, as shown by competition studies with asialoorosomucoid *in vivo*. By using glycolipid-laden liposomes of various sizes between 30 and 90 nm, it was demonstrated that particles with a diameter of >70 nm could no longer be recognized and processed by the ASGPr *in vivo*. This threshold size for effective uptake was not related to the physical barrier raised by the fenestrated sinusoidal endothelium, which shields hepatocytes from the circulation, because similar results were obtained by studying the uptake of liposomes on isolated mouse hepatocytes *in vitro*. From these data we conclude that in addition to the species, valency, and orientation of sugar residues, size is also an important determinant for effective recognition and processing of substrates by the ASGPr. Therefore, these data have important implications for the design of ASGPr-specific carriers that are aimed at hepatocyte-directed delivery of drugs and genes.

The hepatic asialoglycoprotein receptor (ASGPr)[1] is a C-type (Ca^{2+}-dependent) lectin that is expressed on the surface of hepatocytes (1) and plays a role in the clearance (endocytosis and lysosomal degradation) of desialylated proteins from the serum (2, 3) as has been shown for cellular fibronectin (4) and all IgA2 allotypes (5). The human functional receptor is a noncovalent heterotetramer composed of two homologous type II membrane polypeptides with 55% sequence identity, generally called HL-1 (hepatic lectin 1) and HL-2, at a 2:2 stoichiometry (6). The ASGPr binds glycoproteins with either nonreducing terminal β-D-galactose (Gal) or *N*-acetylgalactosamine (GalNAc) residues, at which the affinity for GalNAc is approximately 50-fold higher than for Gal (7–9). From studies using mice that are deficient in either the subunit HL-1 (10) or HL-2 (11), it is evident that both polypeptides are necessary for efficient clearance of asialoglycopeptides. In addition to the ASGPr on hepatocytes, a homologous Ca^{2+}-dependent Gal-recognizing receptor that also recognizes GalNAc and fucose is present in the liver on Kupffer cells (galactose particle receptor (GPr), Gal/fucose receptor) (12, 13) and is absent from all other types of macrophages (14, 15).

Each polypeptide subunit of the ASGPr can bind at least a single terminal Gal or GalNAc residue (16), and the affinity of ligands for the ASGPr appears to be governed by the valency of sugar residues and their appropriate spacing. Studies using asialoglycopeptides from naturally occurring glycopeptides (7, 17) and synthetic cluster glycosides (8, 18) have demonstrated that clustering of glycosides greatly enhances the affinity for the receptor through simultaneous occupation of the receptor sites of the polypeptide subunits, at the following binding hierarchy: tetraantennary > triantennary ≫ biantennary ≫ monoantennary galactosides. This effect is dependent on the structural organization of the receptor on the cell membrane, because it is not observed on the isolated receptor (8, 18). In addition to this so-called "cluster effect," Lee *et al.* (19) and Biessen *et al.* (20) have shown that optimal receptor recognition of synthetic cluster glycosides is also determined by appropriate spacing (at least 15 Å) of the sugar residues.

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[1] The abbreviations used are: ASGPr, asialoglycoprotein receptor; ASOR, asialoorosomucoid; BSA, bovine serum albumin; CO, cholesteryl oleate; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle medium; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GPr, galactose particle receptor; LCO, lithocholic oleate; GABA, γ-aminobutyric acid; LCO-Gal_3, (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-Tris$(Gal)_3$; LCO-Tyr-$GalNAc_3$, (3β(oleoylamido)-5β-cholanoyl)-GABA-Tyr-Gly-Tris$(GalNAc)_3$; PBS, phosphate-buffered saline; Chol, cholesterol; mono, monoantennary.

Although the effects of sugar type and valency on the affinity of ligands for the ASGPr are now well established, the effects of ligand size on the binding characteristics to the receptor have still not been fully mapped. Early *in vivo* studies suggested that the ASGPr is mainly responsible for the uptake of small (≤15 nm) particles exposing galactose at relatively low density, such as high density lipoproteins that are lactosylated (21) or provided with galactose-terminated monoantennary (mono-Gal-Chol) (22, 23) and triantennary glycolipids (Tris-Gal-Chol) (24) and galactose-exposing gold particles (25). In contrast, the GPr predominantly recognizes larger galactose-exposing particles (>15 nm) (26–28), such as desialylated rat erythrocytes (29, 30), low density lipoproteins that are lactosylated (26) or provided with mono-Gal-Chol (22, 23) and Tris-Gal-Chol (31), and Tris-Gal-Chol-exposing liposomes (31). The affinity of glycosides for the GPr was shown to increase with particle size to reach a maximum at 15 nm (27). Furthermore, it has been shown that the GPr preferentially recognizes a high density of either fucose or galactose on either proteins (13, 15) or particles (26, 32).

In contrast to these findings, providing low density lipoproteins with lactosaminated Fab fragments of anti-apoB100 antibodies induces a high uptake of low density lipoproteins by the ASGPr *in vivo* (33). We have also recently shown that even larger (30 nm) liposomes may also be specifically taken up by the ASGPr *in vivo*, when provided with a relatively low amount (<10% w/w) of a nonexchangeable Gal-terminated triantennary glycolipid, with an intrinsic affinity for the ASGPr of 100 nM (32). In addition, *in vitro* studies have suggested that the ASGPr may represent a potential pathway of entry for 28-nm hepatitis A virions (34) and 42-nm hepatitis B virions (35) into hepatocytes. These data indicate that particles larger than 15 nm with their sugars presented at a high local surface density (33), at a low overall surface density (26), or at an appropriate spatial orientation (32) can also be taken up by the ASGPr *in vivo*.

The aim of the present study was to assess the intrinsic upper size limit for binding, uptake, and processing of ligands by the ASGPr. For this purpose, we synthesized a novel triantennary glycolipid that shows stable association with lipidic particles because of a highly lipophilic lithocholic oleate (LCO) structure (32, 36) and a predicted high affinity for the ASGPr by virtue of a triantennary GalNAc-terminated glycoside with 20Å spacing of the GalNAc residues (37, 38). Subsequently, we determined the effect of this glycolipid (LCO-Tyr-GalNAc$_3$) on the ASGPr-mediated uptake of differently sized stable unilamellar liposomes (32, 39) *in vivo* and *in vitro*. The data indicate that the novel glycoside displays a high intrinsic affinity for the ASGPr (2 nM). Moreover, we show that the glycolipid can induce effective recognition and uptake of liposomes with a diameter as large as 70 nm by the ASGPr on hepatocytes *in vitro* and *in vivo*, whereas larger particles do not bind to the ASGPr. These findings not only add to the further characterization of the structural requirements of ligands for proper recognition by the ASGPr but also have important implications for the design of particulate systems that are widely exploited for ASGPr-mediated targeting of drugs and genes to hepatocytes (40–42).

MATERIALS AND METHODS

Animals—10–12-Week-old male C57Bl/6KH mice weighing 24–28 g and Wistar rats weighing 250–300 g (from Broekman Instituut BV, Someren, The Netherlands) fed *ad libitum* with regular chow were used for the *in vivo* experiments.

Chemicals—[1α,2α-^{3}H]Cholesteryl oleate ([^{3}H]CO) and ^{125}I (carrier-free) in NaOH were purchased from Amersham Pharmacia Biotech. Egg yolk phosphatidylcholine (lipoid E PC; 98%) was from Lipoid (Ludwigshafen, Germany). Galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* (crude) and collagenase (EC 3.4.24.3) from *Clostridium histolyticum* (type IV) were from Sigma. Cholesteryl oleate (CO; 97%) was from Janssen (Beersse, Belgium), and Percoll® was from Fluka (Buchs, Switzerland). 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt, horseradish peroxidase type II (200 units/mg), Precipath® L, EDTA, and collagen S (type I) from calf skin were from Roche Molecular Biochemicals. Ketamine (HCl salt, 100 mg/ml) was from Eurovet (Bladel, The Netherlands). Hypnorm (0.315 mg/ml of fentanyl citrate and 10 mg/ml of fluanisone) and thalamonal (0.05 mg/ml of fentanyl and 2.5 mg/ml of droperidol) were from Janssen-Cilag Ltd. (Saunderton, UK). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were from Molecular Probes (Leiden, The Netherlands). Asialoorosomucoid (ASOR) was prepared by enzymatic desialylation (approximately 70%, as judged by the extent of sialic acid release) of human α_1-acid glycoprotein (orosomucoid) from Cohn Fraction VI (99%) from Sigma as described (43). Multiwell cell culture dishes were from Costar (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from Flow Laboratories (Irvine, UK). All other chemicals were of analytical grade.

Synthesis and Characterization of Glycolipids—The synthesis of the ether-linked triantennary galactoside Z-Gly-Tris(Gal)$_3$ (Gal$_3$; M_r =1484) and its γ-aminobutyric acid (GABA)-mediated coupling product with the steroid structure 3α-oleoyloxy cholenic acid, leading to the bifunctional glycolipid (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-Tris-(Gal)$_3$ (LCO-Gal$_3$; M_w 2058) (see Fig. 1*A*) has been recently reported in full detail (32). A novel triantennary *N*-acetylgalactosamine-terminated cluster (Z-Tris(GalNAc)$_3$; M_w 1532) has been synthesized and conjugated with a nearly identical steroid structure via a tyrosine residue to allow for trace labeling with ^{125}I, yielding (3β(oleoylamido)-5β-cholanoyl)-Tyr-Gly-Tris(GalNAc)$_3$ (LCO-Tyr-GalNAc$_3$; M_w 2182) (see Fig. 1*B*). The synthesis of this glycolipid will be described in full detail elsewhere. The homogeneity and identity of both glycolipids has been fully established by high pressure liquid chromatography, NMR spectroscopy, and mass spectroscopy. The freeze-dried glycolipids were dissolved in PBS at a final concentration of 25–50 μg/μl and stored at −80 °C under argon before use. Their stability (which exceeded 12 months) was routinely checked by thin layer chromatography (*n*-butanol, *n*-propanol, 25% NH_4OH, and H_2O 15:40:30:15 v/v/v/v, or isopropanol and 25% NH_4OH 1:1 v/v) and subsequent visualization of carbohydrate and cholesterol moieties by charring with H_2SO_4 and ethanol (1:4 v/v) and $MnCl_2$ (44), respectively.

Radiolabeling of LCO-Tyr-GalNAc$_3$ and ASOR—LCO-Tyr-GalNAc$_3$ was radioiodinated with carrier-free ^{125}I at pH 7.4 using a Iodogen-coated (10 μg) reaction tube, and ASOR at pH 10.0 according to the ICl method (45), respectively. Free ^{125}I was removed by Sephadex G-50 medium gel filtration. The radioiodinated glycolipid migrated as a single band on TLC (*n*-butanol, *n*-propanol, 25% NH_4OH, and H_2O 15:40:30:15 v/v/v/v) as determined by imaging, and more than 98% of the radiolabel in ASOR was 10% trichloroacetic acid-precipitable. The specific activities of LCO-Tyr-GalNAc$_3$ and ASOR were 1300–4300 dpm/ng of glycolipid and 260 dpm/ng of protein, respectively.

Protein Assay—Protein concentrations were determined according to Lowry *et al.* (46) using BSA as a standard.

In Vitro Binding to Hepatocytes—Hepatocytes were isolated from anesthetized rats or mice by perfusion of the liver with collagenase (type IV, 0.05%, w/v) for 10 min at 37 °C according to the method of Seglen (47) as detailed earlier (27). The cells were ≥99% pure as judged by light microscopy, and their viabilities were ≥95% (rat) and ≥80% (mouse) as determined by 0.2% trypan blue exclusion. Hepatocytes were incubated (2 h at 4 °C) in DMEM containing 2% BSA (1×10^6 cells/ml) with 5 nM ^{125}I-ASOR in the presence of increasing amounts of unlabeled galactose (0.2–200 mM), Z-Gly-Tris(Gal)$_3$ (1–1000 nM), or Z-Tyr-Gly-Tris(GalNAc)$_3$ (0.2–200 nM) under gentle shaking in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm. After incubation, the cells were pelleted by centrifugation (1 min at 50 *g*), and unbound ^{125}I-ASOR was removed by washing twice with ice-cold 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ (Tris-buffered saline), pH 7.4, containing 0.2% BSA and once with Tris-buffered saline without BSA. The cell pellet was lysed in 0.1 N NaOH, the radioactivity and protein content was measured, and ^{125}I-ASOR binding was calculated (dpm/mg of cell protein). Nonspecific binding was determined in the presence of 100 mM GalNAc. Displacement binding data were analyzed according to a single-site binding model. Inhibition curves were calculated by nonlinear regression analysis (GraphPad, ISI Software, Philadelphia, PA).

Preparation and Characterization of Liposomes—Liposomes (mean diameters, 30, 50, and 70 nm) were prepared by sonication as described (39). In short, egg yolk phosphatidylcholine (25 mg), CO (1 mg), and

[^{3}H]CO (50–100 μCi) were hydrated in 10 ml of 0.1 M KCl, 10 mM Tris-HCl, pH 8.0, and subsequently sonicated at 54 °C using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 18 μm output. Alternatively, liposomes (mean diameters, 50 and 90 nm) were prepared after hydration of the lipids in 2.0 ml of buffer and multiple extrusion (11 times) at 54 °C through 50- and 100-nm Whatman Nuclepore® (Pleasanton, CA) polycarbonate filters, respectively, using a Liposofast-Pneumatic (Avestin Inc., Ottawa, Canada) (48). All liposomes were purified and concentrated (1.014 g/ml) by density gradient ultracentrifugation according to Redgrave *et al.* (49) using NaCl/KBr/EDTA density solutions in a Beckman SW 40 Ti rotor at 40,000 rpm for 18–22 h at 4 °C. Particle sizes were determined by photon correlation spectroscopy (Malvern 4700 C System, Malvern Instruments, Malvern, UK) at 27 °C and a 90° angle between laser and detector. Sonication for 60, 15, and 10 min resulted in liposomes with mean particle diameters of 29.4 ± 2.2, 55.7 ± 0.9, and 72.3 ± 3.6 nm (mean ± S.D.; n = 3, 2, and 3) that were homogeneous with respect to size (polydispersities of 0.14–0.17, 0.28–0.29, and 0.26–0.27). Extrusion led to liposomes of 48.3 nm (50 nm filter; n = 1) and 90.3 ± 6.1 nm (100 nm filter; mean ± S.D.; n = 4) with polydispersities of 0.15 and 0.11–0.15, respectively. When indicated, liposomes were labeled with 1% (w/w) DiO or DiI by adding 0.25 mg from 10 mg/ml stock solutions in $CHCl_3$:CH_3OH (1:1 v/v) before hydration of lipids. The phosphatidylcholine and cholesterol ester contents were determined with the Roche Molecular Biochemicals enzymatic kits for phospholipid and cholesterol, respectively. Precipath® L was used as an internal standard. The particles were stored at 4 °C under argon and used for characterization and metabolic studies within 7 days following preparation, in which period no physicochemical changes occurred.

Association of LCO-Tyr-GalNAc$_3$ with Liposomes—Liposomes (100 μg of phospholipid) were incubated (30 min at 37 °C) with (radioiodinated) glycolipid in PBS, pH 7.4. The mixtures were subjected to 0.75% (w/w) agarose gel electrophoresis at pH 8.8, and the resulting gels were stained for lipid using Sudan Black. Radioactivity was visualized by imaging using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA). The electrophoretic mobility (R_f) of the Coomassie Brilliant Blue-stained liposomes (0.18 ± 0.01) was determined relative to the front marker bromphenol blue. Alternatively, incubation mixtures (50 μl) were subjected to fast protein liquid chromatography (SMART System; Amersham Pharmacia Biotech) using a Superose® 6 (PC 3.2/30) column at a flow rate of 50 μl/min and with PBS, 1 mM EDTA, 0.02% NaN_3, pH 7.4, as eluent. The galactose content of the collected fractions was determined using a galactose oxidase assay (recovery, 85–100%). In short, samples were incubated in the dark (30 min at room temperature) with 0.9 mM 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt, 66.5 milliunits/ml peroxidase, 2.2 units/ml galactose oxidase, 0.1 M KP_i buffer, pH 7.0, and the absorbance was measured at 405 nm. LCO-Tyr-GalNAc$_3$ was used as a standard. The number of associated glycolipid molecules/30-nm particle was calculated assuming 7.62×10^{12} liposomes/mg of phospholipid (39).

Liver Uptake and Serum Decay of Liposomes in Mice—Mice were anesthetized by subcutaneous injection of a mixture of ketamine (120 mg/kg body weight), thalamonal (0.03 mg/kg fentanyl and 1.7 mg/kg droperidol), and hypnorm (1.2 mg/kg fluanisone and 0.04 mg/kg fentanyl citrate), and the abdomens were opened. [^{3}H]CO-labeled liposomes (100 μg of phospholipid) were injected via the inferior vena cava, after previous incubation (30 min at 37 °C) with PBS or the indicated amounts of glycolipid. When indicated, mice received a preinjection of ASOR (25 mg/kg) at 1 min before injection of the particles. At the indicated times, blood samples (<50 μl) and liver lobules were taken and processed as described in detail (50). At 30 min after injection, the mice were sacrificed, and their livers and spleens were excised and weighed. Radioactivity in duplicate serum samples of 10 μl was counted in 2.5 ml of Emulsifier Safe (Packard Instrument Co.). The total serum volume of C57Bl/6KH mice was 1.068 ± 0.066 ml (50). Radioactivity in liver samples and spleens was counted in 15 ml of Hionic Fluor (Packard Instrument Co.) after solubilization of the organs in 500 μl of Soluene-350® (Packard) for 5 h at 65 °C. Radioactivity values are corrected for the serum radioactivity (liver, 84.7 μl/g wet weight; spleen, 64.6 μl/g wet weight) present at the time of sampling (50).

In Vitro Uptake of Fluorescently Labeled Liposomes by Mouse Hepatocytes—Mouse hepatocytes were isolated from anesthetized mice as described above and subjected to Percoll® gradient centrifugation to discard nonviable cells. The cells (viability >99% as judged from 0.2% trypan blue exclusion) were attached to collagen S-coated (3.87 μg/cm^2) 2.5-cm glass coverslips in 9.6-cm^2 6-well dishes (1×10^6 cells/well) by culturing in DMEM + 10% fetal calf serum (3–4 h at 37 °C). The coverslips were washed to remove unbound cells and transferred to a

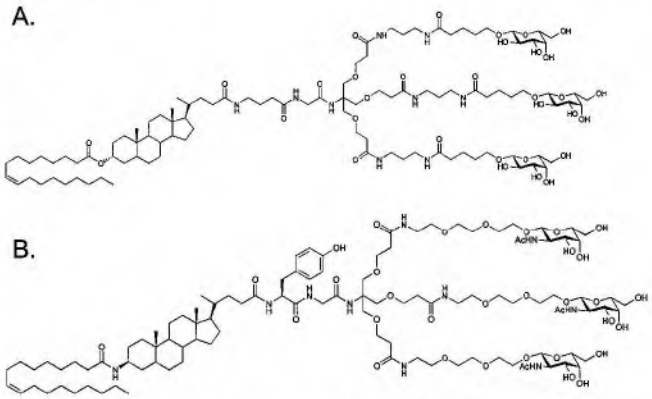

FIG. 1. **Chemical structures of glycolipids.** *A*, (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-Tris(Gal)$_3$ (LCO-Gal$_3$). *B*, (3β(oleoylamido)-5β-cholanoyl)-GABA-Tyr-Gly-Tris(GalNAc)$_3$ (LCO-Tyr-GalNAc$_3$).

Zeiss IM-35 inverted microscope (Oberkochen, Germany) with a Zeiss plan apochromatic 63×/1.4 NA oil objective and fitted with a temperature-controlled incubation chamber, which was equipped with a Bio-Rad 600 MRC confocal laser scanning microscopy system. The cells were incubated (20 min at 37 °C) in DMEM with 2% BSA with DiO-labeled 30-nm liposomes and/or DiI-labeled 90-nm liposomes (200 μg of phospholipid/ml), after previous incubation with 5% (w/w) LCO-Tyr-GalNAc$_3$ or PBS in the absence or presence of 100 mM GalNAc. Subsequently, the cells were washed twice with DMEM with 2% BSA to remove unbound particles, and (intra)cellular localization of DiO and DiI was visualized during further incubation at 37 °C.

In Vitro Association of Radioactively Labeled Liposomes with Mouse Hepatocytes—Mouse hepatocytes were isolated from anesthetized mice as described above, and viable cells were harvested by Percoll® gradient centrifugation. The cells (1×10^6) were incubated at 37 °C in 0.5 ml of DMEM with 2% BSA with [^{3}H]CO-labeled 30-nm (sonicated), 50-nm (extruded), 70-nm (sonicated), and 90-nm (extruded) liposomes (240 μg of phospholipid/ml) with or without previous incubation (30 min at 37 °C) with LCO-Tyr-GalNAc$_3$ (5% w/w) in the absence or presence of 100 mM GalNAc. The incubations were performed in plastic containers (Kartell, Milan, Italy) in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm, with brief oxygenation every 60 min. After incubation, the cells were cooled to 0 °C and pelleted (1 min at 50 *g*), and unbound particles were removed by washing twice with ice-cold Tris-buffered saline, pH 7.4, containing 0.2% BSA, and once with Tris-buffered saline without BSA. The cell pellet was lysed in 0.1 N NaOH, the radioactivity and protein content were measured, and association of liposomes was calculated as μmol phospholipid/mg of cell protein.

RESULTS

Affinity of GalNAc-terminated Triantennary Cluster for the Hepatic ASGPr—We determined the affinity of the newly synthesized triantennary Gal-terminated cluster glycoside Z-Gly-Tris(Gal)$_3$ (Fig. 1*A*) and the GalNAc-terminated cluster glycoside Z-Gly-Tris(GalNAc)$_3$ (Fig. 1*B*) for the rat and murine ASGPr by determining the ability of the cluster glycosides to compete for the binding of the high affinity ligand ^{125}I-ASOR to isolated hepatocytes *in vitro* (Fig. 2). Both galactosides displayed competitive inhibition of ASOR binding, as shown by monophasic inhibition curves with a Hill coefficient close to unity. In agreement with earlier studies (8, 51), galactose was only marginally capable of inhibiting ^{125}I-ASOR binding to rat (K_i 4.3 ± 0.8 mM) and mouse (K_i 3.6 ± 1.0 mM) hepatocytes. The clustered presentation of Gal residues in Z-Gly-Tris(Gal)$_3$ increased the potency approximately 40-fold (K_i 100 ± 1 nM; mean ± S.E.; n = 3). Replacement of Gal by GalNAc in Z-Gly-Tris(GalNAc)$_3$ caused a further 50-fold increased affinity (2.1 ± 0.3 nM and 2.7 ± 1.0 nM toward rat and mouse hepatocytes, respectively), which is in agreement with observations from Lee and Lee (37, 38).

Interaction of Glycolipids with Liposomes—To investigate the interaction of LCO-Tyr-GalNAc$_3$ with the differently

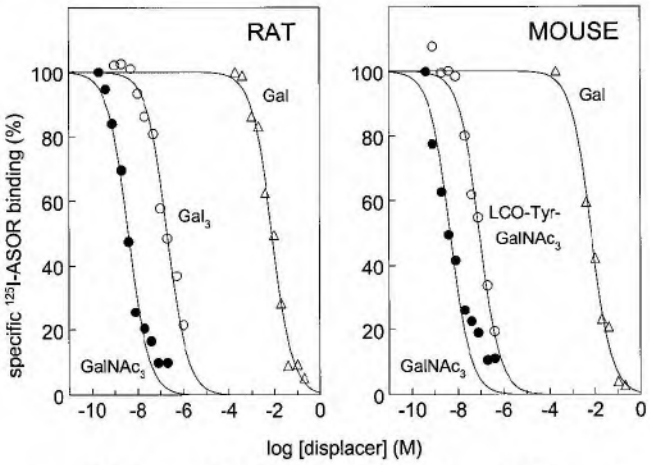

FIG. 2. **Inhibition of ASOR binding to hepatocytes by triantennary galactosides.** Freshly isolated rat (*left panel*) and mouse (*right panel*) hepatocytes (approximately 1×10^6 cells/ml) were incubated with 5 nM ^{125}I-ASOR (2 h at 4 °C) in the absence or presence of unlabeled galactose (△, 0.2–200 mM) and the galactosides Gal_3 (○, 1–1000 nM) or $GalNAc_3$ (●, 0.2–200 nM). Binding is plotted as percentage of specific binding, which is defined as the difference in ligand binding in the absence (total binding) and presence (nonspecific binding) of 100 mM *N*-acetylgalactosamine.

sized egg yolk phosphatidylcholine:CO liposomes, we first examined the effect of the glycolipid on the electrophoretic pattern of the liposomes (Fig. 3). Incubation of 30-nm liposomes with radioiodinated glycolipid resulted in an LCO-Tyr-$GalNAc_3$ concentration-dependent reduction of the electrophoretic mobility of the liposomes ($R_f = 0.18 \pm 0.01$) (Fig. 3*A*). Incorporation of the glycolipid was evidenced by comigration of the radiolabeled glycolipid with the liposomes (Fig. 3*B*). Identical patterns were obtained using liposomes of 55, 70, and 90 nm, indicating a similar extent of glycolipid incorporation (not shown). The incorporation capacity of 30-nm liposomes for LCO-Tyr-$GalNAc_3$ was determined by separation of the liposome-bound glycolipid from the free glycolipid with high resolution by size exclusion chromatography on a Superose 6 column (Fig. 3*C*). LCO-Tyr-$GalNAc_3$ elutes at excellent yield (>95%) at an elution volume of 1.53 ml, indicating that the glycolipids form stable micelles with a size slightly larger than that of human high density lipoproteins (8–10 nm; $V_e = 1.58$ ml), and can easily be separated from the relatively large liposomes ($V_e = 0.90$ ml). Incubation of liposomes with LCO-Tyr-$GalNAc_3$ (5, 10, 25, and 50%, w/w) led to incorporation of 160, 200, 360, and 415 glycolipid molecules/particle, respectively. Incorporation of the glycolipid did not substantially alter the liposomal size, as judged from Sephacryl S-1000 elution profiles (not shown).

Liver Uptake and Serum Decay of (Glycolipid-laden) Liposomes in Mice—Because both glycolipids show similar incorporation characteristics into liposomes, the glycolipid-induced liver uptake of 30-nm liposomes was evaluated for LCO-Tyr-$GalNAc_3$ and LCO-Gal_3. Upon intravenous injection into mice, the [^{3}H]CO-labeled liposomes showed a low uptake by the liver (7.7 ± 0.4% of the injected dose at 30 min after injection) and a high remaining fraction in the serum (81.3 ± 2.1%), as a consequence of their low affinity for the reticuloendothelial system (Fig. 4) (32, 39). In accordance with previous observations obtained with LCO-Gal_3 (32), incubation of the liposomes with LCO-Tyr-$GalNAc_3$ dose-dependently accelerated their serum clearance in a monophasic manner (Fig. 4, *right panel*), indicating that the glycolipid firmly associates with the particles because of its highly hydrophobic moiety and does not readily redistribute to serum lipoproteins. The increased serum

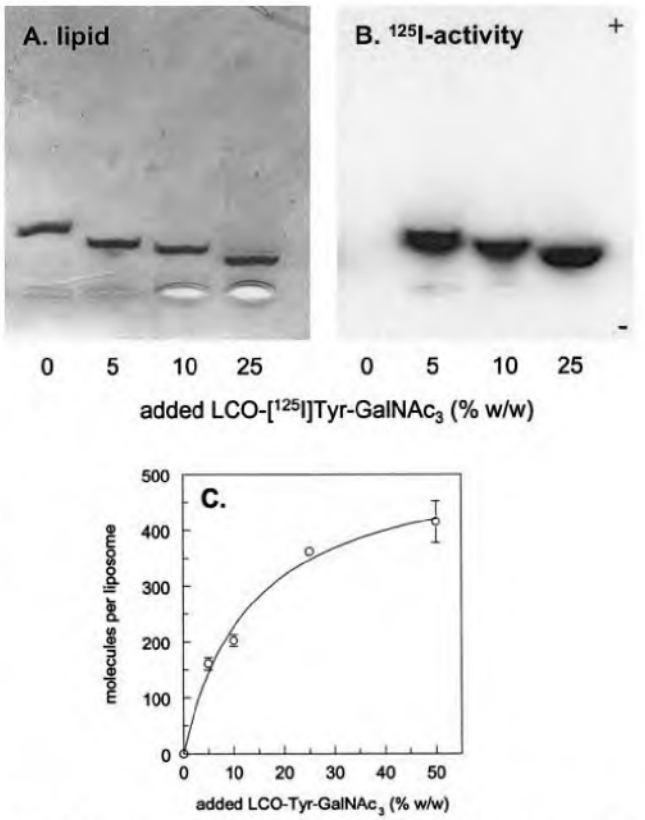

FIG. 3. **Association of LCO-Tyr-$GalNAc_3$ with liposomes.** Liposomes (30 nm; 100 μg of phospholipid) were incubated (30 min at 37 °C) with LCO-[^{125}I]Tyr-$GalNAc_3$ (0, 5, 10, and 25%, w/w phospholipid) and subjected to electrophoresis in a 0.75% agarose gel. Subsequently, the liposomes were stained for lipid (*A*), and glycolipid-associated ^{125}I radioactivity was visualized by imaging (*B*). The anode and cathode are indicated by + and −, respectively. Alternatively, liposomally incorporated glycolipid was separated from unincorporated glycolipid by fast protein liquid chromatography, the galactose content of the resulting fractions was determined, and the number of glycolipid molecules per particle was calculated (*C*).

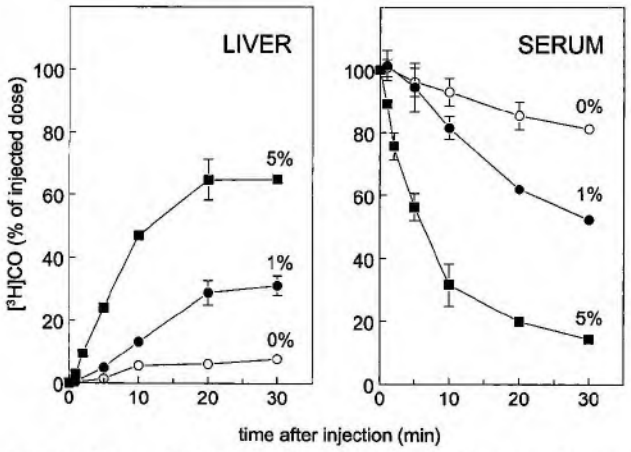

FIG. 4. **Effect of LCO-Tyr-$GalNAc_3$ on the liver uptake and serum decay of liposomes in mice.** [^{3}H]CO-labeled 30-nm liposomes (100 μg of phospholipid) were injected intravenously into anesthetized C57Bl/6 mice without (○) or with previous incubation (30 min at 37 °C) with 1% (w/w) (●) or 5% (w/w) (■) of LCO-Tyr-$GalNAc_3$. At the indicated times, the liver uptake (*left panel*) and serum decay (*right panel*) were determined. The liver values are corrected for entrapped serum radioactivity. The values are the means ± variation of two experiments.

clearance of the liposomes was mainly caused by uptake by the liver, which was dose-dependently enhanced via 31.2 ± 3.0% (1%, w/w; $p < 0.05$) to 65.1 ± 0.3% (5%, w/w; $p < 0.0001$) of the

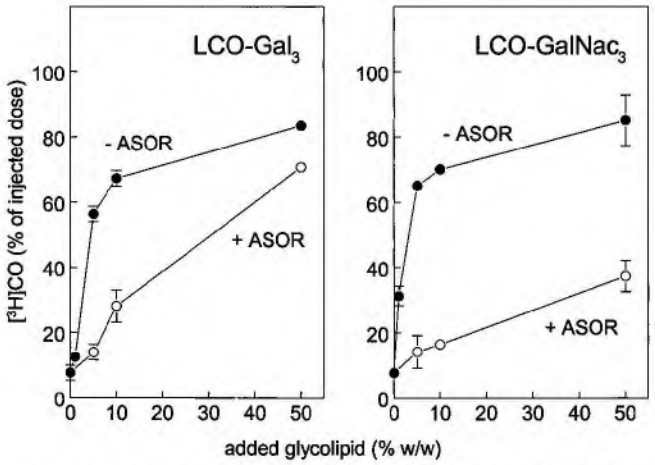

FIG. 5. **Involvement of ASGPr in the glycolipid-induced liver uptake of liposomes.** [^{3}H]CO-labeled 30-nm liposomes (100 μg of phospholipid) were injected intravenously into anesthetized C57Bl/6 mice without or with previous incubation (30 min at 37 °C) with 1, 5, 10, and 50% (w/w) of LCO-Gal$_3$ (*left panel*) or LCO-Tyr-GalNAc$_3$ (*right panel*). At 30 min after injection of the liposomes, the liver uptake was determined without (○) or with (●) previous administration of ASOR (25 mg/kg). The liver values are corrected for serum radioactivity and represent the means ± variation of two experiments.

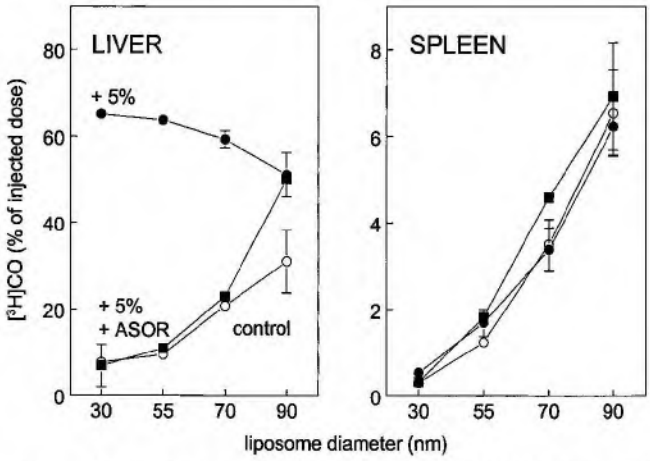

FIG. 6. **Effect of liposomal size on LCO-Tyr-GalNAc$_3$-induced uptake by the liver.** [^{3}H]CO-labeled 30-, 55-, 70-, and 90-nm liposomes (100 μg of phospholipid) were injected intravenously into anesthetized C57Bl/6 mice without (○) or with (● and ■) previous incubation (30 min at 37 °C) with 5% (w/w) of LCO-Tyr-GalNAc$_3$. At 30 min after injection, the mice were sacrificed, and the uptake by the liver (*left panel*) and spleen (*right panel*) were determined, without (○ and ●) or with (■) previous injection of ASOR (25 mg/kg) at 1 min before injection of the liposomes. The values are corrected for serum radioactivity and represent the means ± variation of two experiments.

injected dose at 30 min after injection (Fig. 4).

The effect of the 50-fold higher ASGPr affinity of LCO-GalNAc$_3$ as compared with LCO-Gal$_3$ on the extent of the glycolipid-induced liver uptake of the 30-nm liposomes was addressed by determining the liver uptake of liposomes after incubation with increasing amounts of both glycolipids (Fig. 5). A differential effect of the glycolipids on total liver uptake of the liposomes could predominantly be detected at low incorporation levels. Whereas at 1% (w/w) LCO-Gal$_3$ did not affect the liver uptake, indicating that a threshold loading of liposomes is necessary for inducing affinity for the liver, a 4-fold increased uptake ($p < 0.05$) could already be detected using the same amount of LCO-Tyr-GalNAc$_3$.

Although the effect of the increased ASGPr affinity of LCO-Tyr-GalNAc$_3$ on the total uptake of liposomes by the liver may be limited, a large effect was observed on the ASGPr specificity of the liposomes. This was determined by a preinjection of ASOR, which specifically blocks ASGPr-mediated uptake by hepatocytes, but not GPr-mediated uptake by Kupffer cells (24, 31). At low amounts of LCO-Gal$_3$ ($\leq$ 5%, w/w), the glycolipid-induced liver uptake could be completely blocked by a preinjection of ASOR. However, at higher LCO-Gal$_3$ concentrations, the effect of ASOR on the induced uptake rapidly declined, indicating an almost complete shift in uptake from hepatocytes to Kupffer cells at 50% (w/w). This can be explained by a high surface density of galactose residues that are readily recognized by the GPr (26, 32) (Fig. 5, *left panel*). In contrast, preferential uptake of the liposomes by the ASGPr was observed even at high LCO-Tyr-GalNAc$_3$ concentrations, because a high degree of inhibition (*i.e.* 62%) of the liver uptake by the ASGPr competitor could still be detected at 50% (w/w) (Fig. 5, *right panel*). Apparently, the very high affinity of LCO-Tyr-GalNAc$_3$ for the ASGPr overrules the stimulating effect of a high glycoside surface density on the induced uptake by the GPr.

Size-dependent Association of Liposomes to the ASGPr in Mice—Because it is now evident that LCO-Tyr-GalNAc$_3$ is superior to LCO-Gal$_3$ in its capacity to selectively stimulate the ASGPr-mediated uptake of liposomes *in vivo*, LCO-Tyr-GalNAc$_3$ (5% w/w) was used to evaluate the effect of liposomal size on the ASGPr-mediated uptake by hepatocytes (Fig. 6). As expected from the size-dependent enhanced affinity of liposomes for the reticuloendothelial system, both the hepatic and splenic uptake of the ligand-deficient liposomes increased with increasing liposomal diameter. LCO-Tyr-GalNAc$_3$ was able to induce the liver uptake of liposomes irrespective of their size, although the extent of liver uptake slightly decreased with increasing particle size. Importantly, the glycolipid-induced liver uptake of 30-, 55-, and 70-nm particles (prepared by sonication) could be almost completely blocked by preinjection of ASOR, indicating that the uptake is fully mediated by the ASGPr. In contrast, although the liver association of the 90-nm liposomes (prepared by extrusion) also seemed to be enhanced by LCO-Tyr-GalNAc$_3$, ASOR was unable to compete for the liver uptake, indicating that the ASGPr is not involved (Fig. 6). The inability of ASOR to block the glycolipid-induced liver uptake of these large liposomes cannot be ascribed to an increased affinity of these liposomes for the ASGPr. At a concentration of 0.4 μM, LCO-Tyr-GalNAc$_3$ micelles inhibited the ASOR binding to hepatocytes for 80.6% (Fig. 2*B*), and a similar inhibition (77.3%) could be detected for glycolipid-laden 30-nm liposomes (5%, w/w). In contrast, glycolipid-laden 90-nm liposomes displayed a severely reduced inhibitory activity (20.0%), indicating a much lower affinity of these glycolipid-containing liposomes for the ASGPr (not shown). The liver uptake of 50-nm liposomes that were prepared by extrusion (10.2 ± 1.8% of the injected dose at 30 min after injection) was enhanced to a similar extent as compared with the 55-nm sonicated liposomes by LCO-Tyr-GalNAc$_3$ (59.3 ± 3.1%) and could also be fully inhibited by a preinjection of ASOR (13.6 ± 1.9%). Therefore, a potentially disturbing effect of preparation method on the *in vivo* characteristics of the liposomes can be excluded. No effects of LCO-Tyr-GalNAc$_3$ were observed on the splenic accumulation of the liposomes, despite the presence of binding sites for galactose-terminated triantennary glycosides in the spleen.[2]

Size-dependent Uptake of Liposomes by the ASGPr on Hepatocytes—The *in vivo* results point to the existence of a particle

[2] P. C. N. Rensen, L. A. J. M. Sliedregt, T. J. C. Van Berkel, E. A. L. Biessen, unpublished observations.

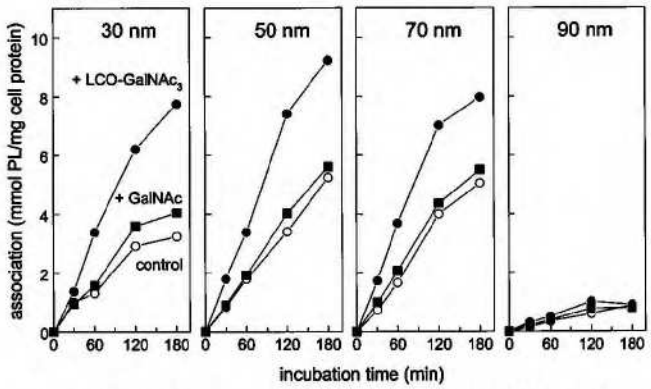

FIG. 7. **Effect of liposomal size on LCO-Tyr-GalNAc$_3$-induced association with isolated mouse hepatocytes.** Freshly isolated mouse hepatocytes were incubated at 37 °C with [^{3}H]CO-labeled 30-, 50-, 70-, or 90-nm liposomes (240 μg of phospholipid/ml) without (○) or with (● and ■) 5% (w/w) of LCO-Tyr-GalNAc$_3$. Incubation with LCO-Tyr-GalNAc$_3$-laden liposomes was done in the absence (●) or presence (■) of 100 mM GalNAc. At the indicated times, the cells were washed and lysed, and cell protein was determined. The values are the means of duplicate incubations.

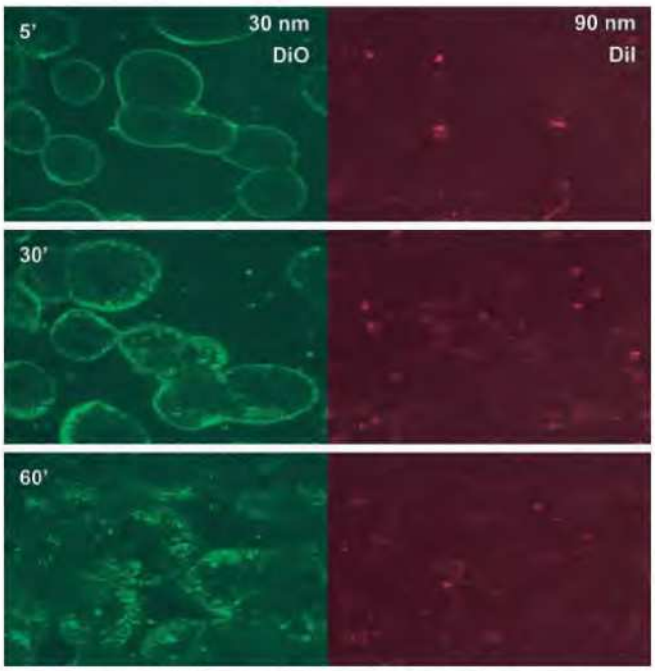

FIG. 8. **Effect of liposomal size on LCO-Tyr-GalNAc$_3$-induced uptake by isolated mouse hepatocytes.** Freshly isolated mouse hepatocytes were cultured (3–4 h at 37 °C) in DMEM with 10% fetal calf serum and coincubated (20 min at 37 °C) in DMEM with 2% BSA with fluorescently labeled 30-nm DiO-labeled (*left panels*) and 90-nm DiI-labeled (*right panels*) liposomes (200 μg of phospholipid/ml) (preincubated with 5% (w/w) of LCO-Tyr-GalNAc$_3$). The cells were washed to remove unbound particles and further incubated at 37 °C. After 5 min (*top panel*), 30 min (*middle panel*), and 60 min (*bottom panel*), localization of DiO (excitation 488 nm, *left panels*) and DiI (excitation 543 nm, *right panels*) was determined by confocal laser scanning microscopy.

size limit (<90 nm) below which liposomes still can associate with the hepatic ASGPr. Because this diameter concurs with the size of the fenestrae that are present in the sinusoidal endothelium of the liver (approximately 100 nm) (52), we subsequently evaluated whether this physiological barrier may have contributed to the observed size effects *in vivo*. Therefore, liposomal uptake experiments were performed using freshly isolated mouse hepatocytes *in vitro* (Figs. 7 and 8).

To quantify the association of the liposomes via the ASGPr on hepatocytes *in vitro*, hepatocytes were incubated with [^{3}H]CO-labeled particles (Fig. 7). The glycolipid-deficient 30-, 50-, and 70-nm liposomes showed a time-dependent association with the hepatocytes (3.24, 5.23, and 5.04 mmol of phospholipid/mg of cell protein after 180 min of incubation, respectively), which was substantially higher than that of the 90-nm liposomes (0.89 mmol of phospholipid/mg of cell protein). The cellular association (binding and uptake) of the 30-, 50-, and 70-nm liposomes was increased upon the addition of 5% (w/w) LCO-Tyr-GalNAc$_3$ and could be blocked by the presence of an excess of GalNAc. In contrast, the cellular uptake of the 90-nm liposomes was not affected by the glycolipid. The observation that the 50-nm liposomes (synthesized by extrusion) showed a similar glycolipid-dependent cell association as the 30- and 70-nm liposomes (prepared by sonication) excludes the possibility that the absence of an effect of the glycolipid on the cell association of the 90-nm liposomes could be due to a difference in liposomal preparation method.

The observed differences in interaction of small (30 nm) and large (90 nm) liposomes with isolated hepatocytes were also demonstrated by confocal laser scanning microscopy (Fig. 8). Pulse labeling of hepatocytes for 20 min with fluorescently labeled liposomes of 30 nm (DiO) and 90 nm (DiI) that contain 5% (w/w) LCO-Tyr-GalNAc$_3$ resulted in a strong fluorescent lining of the cell surface with the 30-nm liposomes but not the 90-nm particles. This effect is not caused by mutual competition between the differently sized liposomes, because exclusion of the small liposomes from the incubation did not result in an enhanced binding of the 90-nm liposomes (not shown). After removal of unbound particles, the surface-bound 30-nm liposomes were rapidly taken up, with complete internalization observed between 30 and 60 min. Binding and uptake of the liposomes was markedly inhibited in the presence of 100 mM GalNAc or in the absence of the glycolipid (data not shown).

DISCUSSION

So far, the ligand recognition by the ASGPr has been well characterized with respect to sugar preference (GalNAc ≫ Gal) (7–9), optimum ligand valency (tetraantennary > triantennary ≫ diantennary ≫ monoantennary) (8, 18) and sugar spacing (20 Å ≫ 10 Å ≫ 4 Å) (19, 20), but the effect of size on recognition and processing of ligands by the ASGPr has been subject of controversy. Early *in vivo* studies on galactose-terminated glycolipids (22–24, 31, 53) have suggested that small (≤15 nm) galactose-exposing particles are preferentially taken up by the ASGPr on hepatocytes, whereas larger particles (>15 nm) mainly associate with the GPr on Kupffer cells. Because this hypothesis has been disputed by the findings that the hepatitis A virus (28 nm) (34), hepatitis B virus (42 nm) (35), 25-nm low density lipoproteins (33), and 30-nm liposomes (32) may also be internalized by hepatocytes via the ASGPr, the present study was undertaken to conclusively establish the effects of size on processing of globular ligands by the ASGPr.

Taking advantage of the above-mentioned "affinity rules," we have synthesized the triantennary glycoside Z-Tris(GalNAc)$_3$ with a nanomolar affinity for the ASGPr (K_i = 2 nM). This affinity is similar to that of the triantennary glycopeptides YEE(GalNAcAH)$_3$ and YDD(G-ah-GalNAc)$_3$ that have been developed by Lee and Lee (37, 38) and utilized for ASGPr-directed delivery of DNA (54) and oligodeoxynucleoside methylphosphonates (55). To establish firm association with liposomes, the glycoside was coupled to lithocholic oleate, which has already been shown to confer a stable incorporation of antisense oligodeoxynucleotides (36), anthracyclines (56), and glycosides (32) into lipidic particles. Also in this study, a tight association of the Gal$_3$ and GalNAc$_3$-terminated glycolipids with the liposomes was observed, withstanding dissociation in the blood.

Unlike the previously applied cholesterol-coupled glycosides, which induced a biphasic serum decay of lipoproteins as explained by partial glycolipid-induced clearance of injected lipoproteins by the liver in the α-phase, followed by redistribution of glycolipid over the endogenous lipoprotein pool in the β-phase (23, 24, 53), the present glycolipids induced a monophasic clearance of liposomes from the serum upon intravenous injection. The differences between both glycolipids with respect to the lipophilic moiety, the absence or presence of a Tyr moiety, and the sugar type (Gal or GalNAc) apparently do not affect the association of the glycolipid with the liposomes.

As a result of the 50-fold higher ASGPr affinity of the $GalNAc_3$-terminated glycolipid over the Gal_3-terminated glycolipid, an effective targeting of 30-nm liposomes to the ASGPr could already be accomplished at concentrations as low as 1% (w/w), at which the Gal_3-terminated glycolipid had no effect. This amount corresponds to only 36 glycolipid molecules/particle, assuming 7.62×10^{13} liposomes/mg of phospholipid (39). When taking into account that 60% of the phospholipids are located in the outer phospholipid layer (39), it thus appears that approximately 22 molecules of the $GalNAc_3$-terminated glycolipid are sufficient for inducing uptake of liposomes by the ASGPr. The most prominent effect of the higher affinity for the ASGPr, however, involves the considerably enhanced specificity of liposomes for the ASGPr as opposed to the GPr over a wide glycolipid loading range (1–50%, w/w). It can be calculated that at an incorporation of 50% (w/w) of glycolipid, the glycoside occupies only a very restricted surface area (approximately 3 nm^2) (32). In this case, the conformational properties of the individual clusters of the Gal_3-terminated glycolipid, which are of vital importance for high affinity recognition by the ASGPr, may be overruled by the high overall Gal density on the liposomal surface, which has been shown to lead to efficient uptake by the GPr on Kupffer cells (27). Apparently, the preferential uptake of liposomes provided with an equal concentration (*i.e.* 50%, w/w) of the $GalNAc_3$-terminated glycolipid by hepatocytes indicates that, in contrast to Gal clusters, a similarly high density of GalNAc clusters on the particle surface does not impair the specificity for the ASGPr. Therefore, the $GalNAc_3$-exposing glycolipid is very suitable for evaluation of the effect of particle size on recognition and uptake by the ASGPr *in vivo* and *in vitro*. These findings have also important implications for research on liposome-mediated ASGPr-directed drug delivery to hepatocytes. Although galactose is generally utilized as a recognition marker for the ASGPr (57), the present data demonstrate that the specificity for this receptor *in vivo* may be greatly improved by the application of the ligand GalNAc instead.

The hepatic and splenic uptake of the glycolipid-deficient liposomes appeared to be enhanced with increasing particle size. Because an opposite effect was observed with respect to the uptake of glycolipid-deficient liposomes by isolated hepatocytes (30–70 nm *versus* 90 nm) and given the fact that the affinity of particulate carriers for the reticuloendothelial system increases with increasing particle size, it is likely that this size-dependent (glycolipid-independent) liver uptake is exerted by Kupffer cells. At a load of 5% (w/w), the $GalNAc_3$-exposing glycolipid did not stimulate the uptake of liposomes by macrophages in general and splenic macrophages in particular, although C-type Gal/GalNAc-recognizing receptors have been described on extrahepatic macrophages from rats and mice (58–62) in addition to S-type (soluble) Ca^{2+}-independent galactoside-binding proteins (galaptins and galectins) (63, 64). Possibly, the macrophage asialoglycoprotein-binding protein displays a lower affinity toward GalNAc than Gal and lacks the cluster effect that has been observed for the ASGPr (65). Similarly, galectins have been shown to have minimal affinity for triantennary cluster galactosides as compared with C-type lectins (63, 64, 66).

Using LCO-Tyr-$GalNAc_3$ at a load of 5% (w/w), it appeared that liposomes with a size up to 70 nm are effectively recognized by the ASGPr on hepatocytes *in vivo*, whereas the 90-nm particles did not associate with the ASGPr. This phenomenon is not caused by the physical barrier raised by the fenestrated endothelium that shields hepatocytes from the circulation, because similar findings were obtained from liposome uptake experiments by isolated hepatocytes *in vitro*. It is unlikely that these observations are related to a restricted size limit of endosomes formed after ASGPr-mediated endocytosis via clathrin-coated pits, because the average endosome size in mammalian hepatocytes has been reported to be 100 nm (67), with a wide size distribution of 50–350 nm (68). Moreover, as compared with the 30–70-nm particles, the 90-nm liposomes already displayed an impaired binding to isolated hepatocytes. Taking into account that the ASGPr is predominantly diffusely and perhaps inaccessibly distributed within the microvilli clefts at the sinusoidal hepatocyte surface (69–71), it is tempting to assume that penetration of particles between the microvilli may be a limiting factor for ASGPr-mediated uptake, but this possibility cannot be conclusively established from the current experimental set up. Regardless of the precise mechanism, our present data may explain why earlier attempts to efficiently target relatively large liposomes to the ASGPr on hepatocytes have not been successful. For example, the observation that 100-nm liposomes provided with (monoantennary) polyethylene glycol-coupled galactolipids were mainly taken up by Kupffer cells (57) may not only be explained by lack of ASGPr specificity but also by their unfavorable dimensions.

In conclusion, in addition to the sugar preference and cluster effect, we have further elucidated the ligand recognition characteristics of the ASGPr by demonstrating that effective binding and internalization by the receptor is restricted to glycoside-exposing particles with a diameter $\leq$70 nm, whereas larger particles are not recognized. Because of its unique localization, abundance, and high internalization capacity, the ASGPr is widely used as a target for the specific delivery of genes and therapeutic agents to hepatocytes. Therefore, our findings also have important implications for the design of such nonviral gene vectors and drug targeting vehicles with respect to sugar ligand (GalNAc $\gg$ Gal) and particle size ($\leq$70 nm).

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CERTIFICATE OF SERVICE

I hereby certify that on February 2, 2024, I caused the foregoing to be electronically filed with the Clerk of the Court using CM/ECF, which will send notification of such filing to all registered participants.

/s/ Kaitlin E. Maloney
Kaitlin E. Maloney (#6304)